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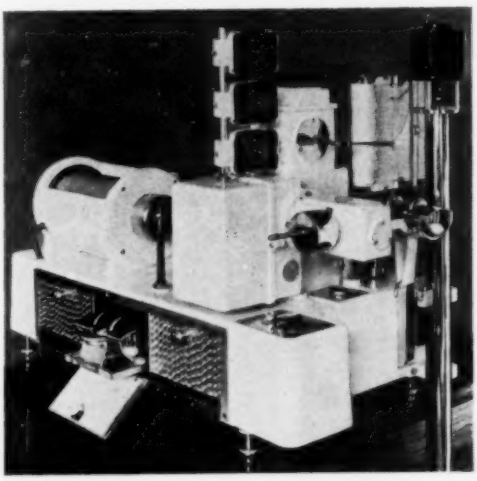

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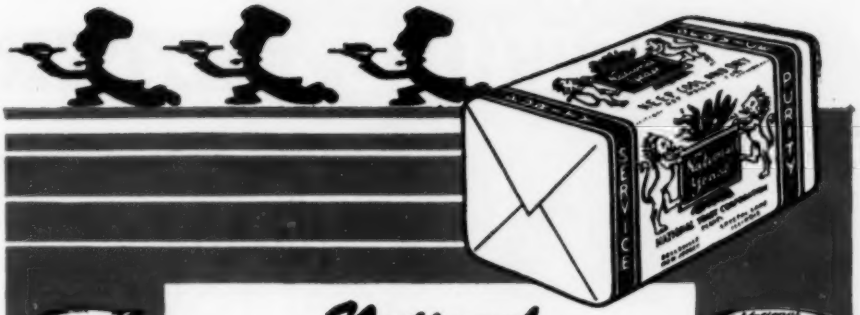

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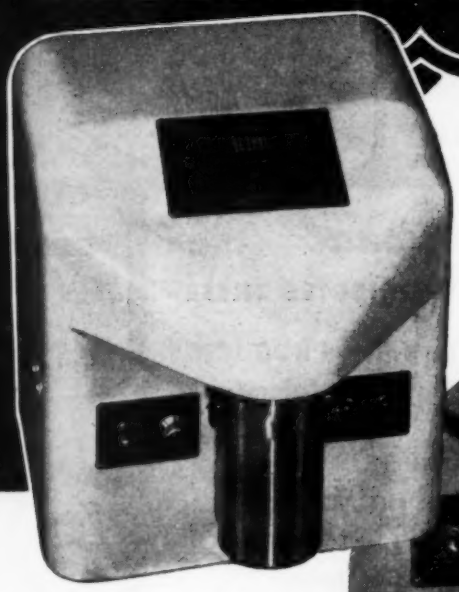

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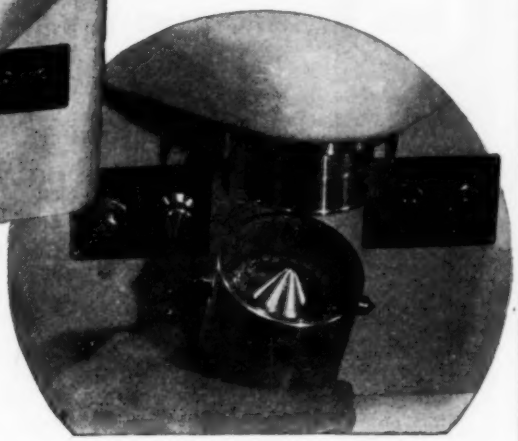

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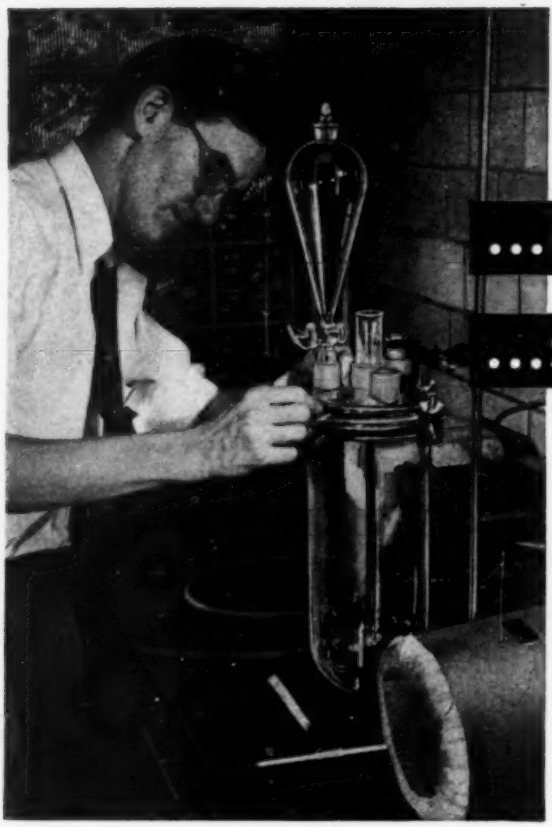

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CEREAL CHEMISTRY

VOL. XXVI MARCH, 1949 No. 2

EFFECTS OF MIXING, SALT, AND CONSISTENCY ON EXTENSOGRAMS [1]

M. H. FISHER, T. R. AITKEN, and J. A. ANDERSON

ABSTRACT

Studies of the effects of initial mixing procedures on extensograms were made with the Hobart, Brabender Farinograph, and Swanson mixers. With the mild mixing action of the Hobart, little change in extensibility (length) or resistance to extension (height) occurs as mixing time is increased, and there is little difference between 45-minute and 135-minute extensograms. With the severe mixing of the Swanson, extensibility decreases and resistance increases with increasing mixing time, and these changes are greater in 135- than in 45-minute extensograms. The farinograph mixer gives intermediate results. Mild mixing is considered to yield extensograms representing inherent properties of the flour and to provide a control procedure for studies of changes in formula or manipulations. Severe mixing yields extensograms from which the response of the flour to mixing can be measured by the differences between 45- and 135-minute curves. Flours from a wide range of wheats differ greatly in both basic properties and in response to mixing.

Increasing the consistency of the dough decreases extensibility and increases resistance. By contrast, increasing the salt concentration increases both extensibility and resistance. Salt also changes the shape of the curve by moving the highest point towards the right. Weak and strong flours respond differentially to added salt, so that comparisons of extensibility depend upon whether this is measured by the total length of the extensogram or by the length to the highest point (greatest resistance). These two measurements are discussed in terms of their probable relation to elastic and viscous deformation during stretching.

As a basic procedure for extensograph tests, the authors have adopted the salt concentration, consistency, and mixing procedure used in their test baking.

Tests made with the Brabender Extensograph can be varied in technique as widely as the baking test. Absorption, formula, type of mixer, and time of mixing can be readily changed. The schedule of dough manipulations, which corresponds to punching in the baking test, can be varied for number, timing, and kind of treatment.

[1] Manuscript received December 14, 1948.
Paper No. 94 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Manitoba, and No. 265 of the Associate Committee on Grain Research (Canada).

Changes can be made in the conditions under which the doughs are rested. The extensograph mechanism can be adjusted to give extensograms of different heights, and it would not be difficult to change the method of stretching in other ways. Various curve dimensions can be measured, and these can be reported in different units. Great scope clearly exists for varying the technique, and it is difficult to select the best procedure for given purposes.

The technique recommended by the Brabender Corporation in the Manual of Instructions appears to represent a relatively arbitrary selection. Munz and Brabender (5, 6, 7) did not use the recommended procedure in any of their comprehensive studies. They employed a wide range of techniques, usually tested several replicate doughs treated in different ways, and generally condensed their data by such methods as plotting the F/E ratio (height/length) against the logarithm of rest period. These investigations have little bearing on the development of a simple and rapid extensograph test. Indeed, the inescapable conclusion appears to be that a large number of extensograph tests are required to obtain useful information about a flour, and that more comprehensive and perspicuous data may well be provided by devoting an equal effort to traditional baking studies.

Other investigators (1, 3, 4) have also deviated from the manufacturer's instructions, and private communications and observations made during visits to various laboratories, on this continent and in the United Kingdom, suggest that a wide range of techniques is now in use. If all the information about varying the technique were published and could be collated, substantial progress in defining the best methods for using the extensograph might well result. This paper is published with that goal in mind.

In this laboratory, the purpose has been to devise the simplest extensograph test or tests that will yield interpretable data on the innate properties of flour. Provided that tests can be made rapidly, the extensograph may well have widespread uses for control in mill and bakeshop, and possibly as a tool for research. Development along either line is inhibited by inadequate information about rudimentary technique. Without this knowledge, it is difficult to find a logical point of departure for more advanced studies or adequate knowledge for interpretation of data that such studies may yield.

This paper presents results of studies of mixing, salt concentration, and consistency. These factors were investigated with flours made from a series of wheats representing a wide range of types so that differential responses of type to change in technique might be demonstrated. Although large numbers of data were accumulated, only sufficient need be presented to illustrate the points discussed. A brief des-

cription of the materials is given in the following section. Separate sections are then devoted to a study of mixing and a study of the effects of varying salt concentration and consistency. As a result of these investigations, certain basic techniques and concepts have been adopted in this laboratory and are discussed in the final section.

Materials

Descriptions of the materials used in these studies are of little importance since any other series of wheats exhibiting a similar range in quality would have been equally suitable. Sixteen wheats of different classes were obtained from various countries, but it is not claimed that all samples represent the average of their class. The wheats were cleaned, and were then milled in an Allis-Chalmers laboratory mill to yield long patent flours of about 71% extraction. The wheats represented and the protein contents of the flours are listed below:

Ref. letter	*Wheat*	*Flour protein, %*
A	High protein Canadian (4 Northern)	16.1
B	Cadet variety (grown in Canada)	14.1
C	Rescue variety (grown in Canada)	13.6
D	Marquis variety (grown in Canada)	13.5
E	No. 1 Northern (average sample)	13.0
F	Amber durum	12.1
G	Garnet variety (grown in Canada)	10.9
H	Plate (Argentine semihard winter)	10.7
I	Kansas hard winter	10.5
J	Alberta red winter	10.3
K	Kansas soft winter	10.1
L	Pacific (U. S.) soft winter	9.7
M	Australian	9.3
N	English	9.2
O	Ontario white winter	9.0
P	Low protein Canadian (3 Northern)	8.6

Effects of Mixing

The manufacturers specify that doughs for extensograph tests be mixed in the farinograph mixer. In a preliminary trial, the amount of water added to a flour containing 2% salt is adjusted so that the center of the farinogram reaches the 500-unit line when the curve attains its maximum height. After adding the required amount of water, the dough for the extensograph test is mixed for 1 minute, rested for 5 minutes, and then mixed until the center of the curve just reaches the 500-unit line. This is variously referred to, by different authors, as mixing to minimum mobility, mixing to maximum dough development, or mixing to dough development time.

Johnson, Shellenberger, and Swanson (3) followed these instructions. Merritt and Bailey (4) varied them by omitting the 5-minute rest period. But Munz and Brabender himself (5) apparently mixed

all flours for 5 minutes, except in their study of extended mixing. Aitken, Fisher, and Anderson (1) also mixed for 5 minutes, but worked at a dough consistency represented by the 600- rather than the 500-unit line. The selection of the farinograph for mixing doughs for extensograph tests appears to be an arbitrary one for which the literature contains no support.

Experimental. Studies were made by mixing doughs for 1, 2, 3, and 4 minutes in the farinograph, the Hobart (medium speed), and the Swanson mixers, using a salt concentration of 2% and a dough consistency of 500 units. After mixing, two 100-g. doughs were scaled, rounded and rolled in the extensograph, rested for 45 minutes at 30°C. and 85% relative humidity, and subsequently stretched. Doughs were

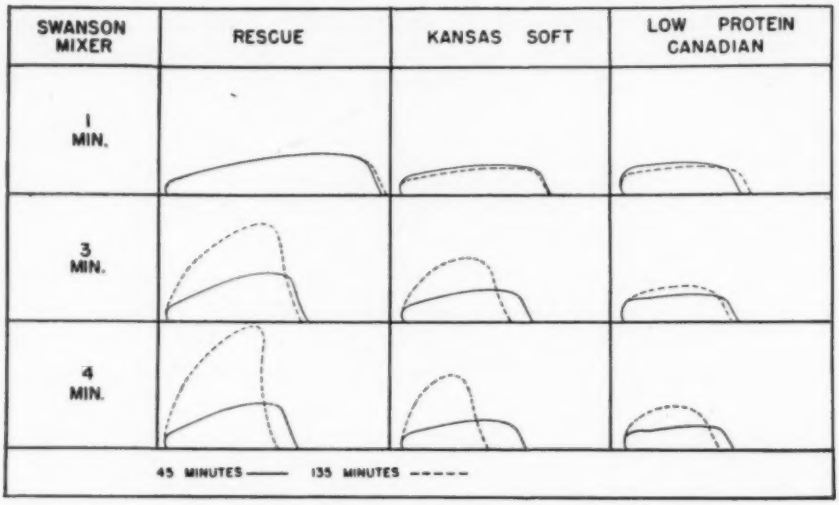

Fig. 1. Extensograms (45 min. solid, 135 min. dotted) showing effects of mixing doughs in the Swanson mixer for different times.

rounded and rolled a second time after 45 minutes but were not stretched. After a further 45 minutes, the doughs were stretched a second time. Extensograms were thus made 45 and 135 minutes after mixing; but the second curve, like the first, was also made 45 minutes after the last rounding and rolling. Six flours were studied, but a selection of the results is adequate to illustrate the principles established.

Because the Swanson mixer has the most severe action on the dough, results obtained with it are exaggerated the most. Reproduction of the extensograms for three of the samples (Rescue, Kansas soft winter, and low-protein Canadian) are shown in Fig. 1. With the 45-minute curve (solid) increasing the mixing time decreases length

and increases height, and this effect is greater for Rescue than for the other two flours. Similar but still greater effects are shown by the 135-minute curves (dotted). Thus, the difference between 45- and 135-minute curves increases with increasing initial mixing, and this response is greatest for the first flour, intermediate for the second, and least for the third.

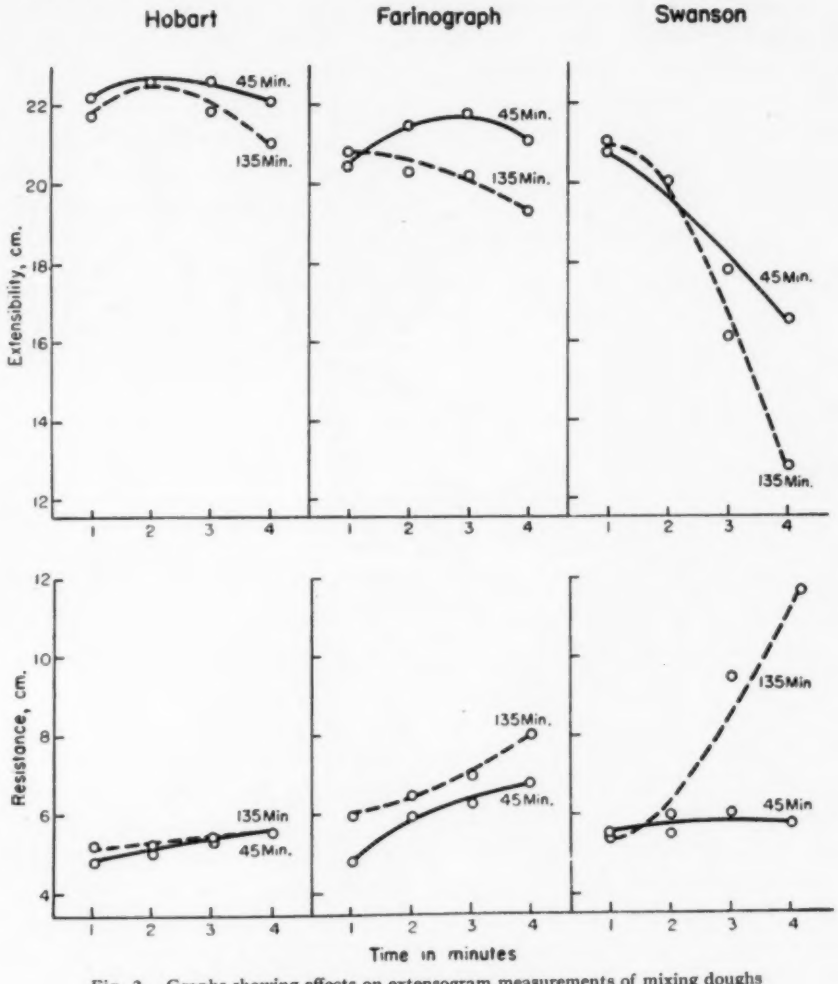

Fig. 2. Graphs showing effects on extensogram measurements of mixing doughs in different mixers. The data are mean values for flours C, E, G, K, L, and P.

These results cannot readily be compared with those of Munz and Brabender (5) because they reported resistance and extensibility as a ratio plotted against the logarithm of rest period. However, their study of extended mixing certainly demonstrated a differential response of flours to mixing time.

The differential effects of the three mixers are illustrated by the graphs in Fig. 2. Extensibility and resistance at 45 minutes (solid lines) and at 135 minutes (dotted lines) are plotted against mixing time, for the mean data obtained with the six flours, C, E, G, K, L, and P. For the Hobart mixer, which has the mildest action, extensogram dimensions remain closely similar for both rest periods as mixing time increases. The farinograph mixer, which has more severe action than the Hobart mixer, shows an increased spread between extensogram dimensions with increased mixing time, and this is exaggerated by the much greater severity of the Swanson mixer. Optima in the upper 45-minute curves for the Hobart and farinograph mixers are interesting; apparently the dough is most extensible when mixed in either mixer for 3 minutes. With the Swanson mixer, maximum extensibility was obtained with minimum mixing time.

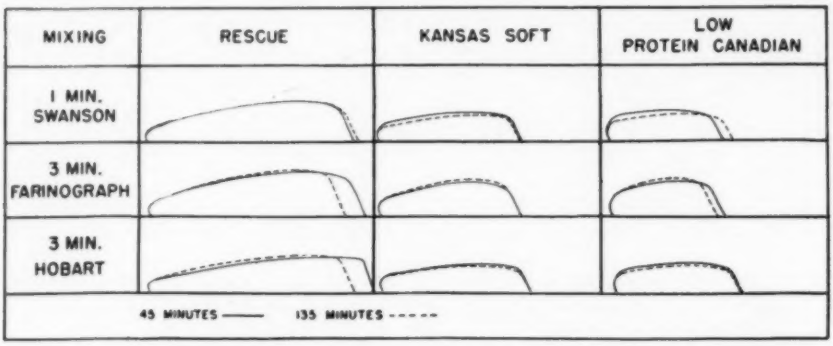

Fig. 3. Extensograms (45 min. solid, 135 min. dotted) showing effects of mixing doughs in different mixers when about the same amount of mixing is done on the dough.

Provided that about the same amount of mixing is done on the dough, the type of mixer is probably unimportant. Fig. 3 shows that 45-minute extensograms afford essentially the same comparisons between the same three flours represented in Fig. 1, irrespective of whether the dough is mixed for 1 minute in the Swanson mixer or for 3 minutes in either the farinograph or the Hobart mixer. Moreover, with these amounts of mixing, the 135-minute extensograms are not widely different from the 45-minute extensograms, though there are some differences which cannot be accounted for merely by experimental error and must therefore be attributed to the mixing.

Mixing to dough development time in the farinograph mixer is compared in Fig. 4 with mixing for 3 minutes in the Hobart mixer using flours A, E, H, P, I, and G. That the three flours in the upper half of the figure exhibit considerable response to mixing is shown by the differences between the 45-minute and the 135-minute extenso-

grams in the top row; farinograph mixing times for these three samples, from left to right, were 6, 6, and 2¼ minutes. When the same samples were mixed for 3 minutes in the Hobart mixer, the first two show their response to mixing by a shorter extensogram at 135 minutes, whereas the extensogram for the Plate flour (H) at 135 minutes shows a decrease in height. The lower half of the figure shows extensograms for three flours that have very little response to mixing. Farinograph mixing times for these flours, from left to right, were 1¾, 3, and 2 minutes. Regardless of whether these three doughs were

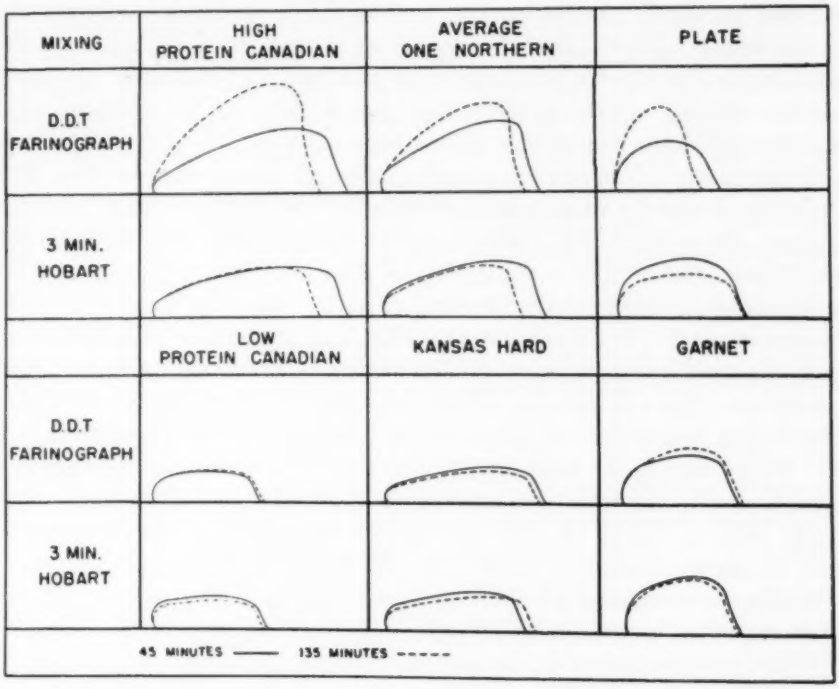

Fig. 4. Extensograms (45 min. solid, 135 min. dotted) showing effects of mixing to development time in the farinograph and for 3 min. in the Hobart mixer.

mixed for a variable time in the farinograph mixer or for 3 minutes in the Hobart mixer, extensograms for 45 minutes and 135 minutes are essentially the same.

Discussion. Whether it is best to vary mixing to meet the requirements of individual flours or to mix all flours equally is debatable. If variable mixing is adopted in accordance with the manual, the treatment should presumably be optimum for each flour, and this supposes that an estimation of optimum treatment, preferably an objective one, is available. Mixing to dough development time in the farinograph

mixer does provide an objective test; but it is questionable whether this procedure assesses optimum mixing treatment for each flour. Observation suggests that some doughs are overmixed. If there is no assurance that each dough can be mixed to given optimum mixing, then all doughs may well be given equal mixing treatment. Otherwise, there is no way of knowing whether results wholly represent intrinsic differences between flours, or are biased by differences in the accuracy with which optimum mixing conditions have been selected.

When all flours are mixed in the same way, the interpretation of the resulting extensograms depends on the severity of the selected mixing method. A distinction may usefully be made between the basic properties exhibited by the dough after mild mixing and the properties the dough may develop through its response to more severe mixing. Mild mixing may be defined as an amount that causes minimum changes in dimensions between 45- and 135-minute extensograms. Results for a wide range of flours suggest that this criterion is met by mixing for 3 minutes in the Hobart mixer. By adjusting the mixing time, other mixers with relatively mild actions will doubtless give similar results. Since 45- and 135-minute curves are essentially the same, only the first need be prepared when this technique is used. Data on extensibility and resistance thus obtained may be considered to represent the basic properties of the flour. The technique thus provides a control procedure with which the effects of modifying manipulative procedure or formula may be compared.

By increasing the time or severity of the initial mixing, the extensograph can be used to measure response to mixing. This response is more pronounced in the 135-minute extensograms. A strict comparison of mixing responses might thus involve comparison of 135-minute extensograms made with the basic procedure and with severe mixing. For many purposes, however, the difference between 45- and 135-minute extensograms made on one dough that has been severely mixed provides an adequate comparative measurement of mixing response.

When the extensograph is used to study the effects on dough properties of some added ingredient, mild mixing may well prove best. For instance, the bromate response, which takes some time to develop, can be measured with 135-minute extensograms. If the mixing is mild, the effect of bromate is negligible after 45 minutes, and the curve thus drawn represents the basic dough properties. Accordingly, the difference between the 45- and 135-minute curves represents the effect of bromate. But if the mixing is severe, the difference between the curves represents a combination of mixing response and bromate response.

As extensograms are normally made at a standard dough consistency, some means of determining the absorption that gives this consistency is required; and the farinograph is extremely convenient for this purpose. But there appears to be no other reason why the farinograph should be used to mix doughs for extensograph tests. For many purposes, the mixer normally used for test baking, or a mixer that closely simulates commercial operations, will represent a more logical choice.

Effects of Varying Salt Concentration and Consistency

The prescribed technique for extensograph tests recommends 2% of salt and a consistency of 500 farinograph units. Except for the use of 600 units for consistency in one investigation (1), this procedure seems to have been generally adopted. Since the salt concentration is higher and the consistency lower than levels generally used in test

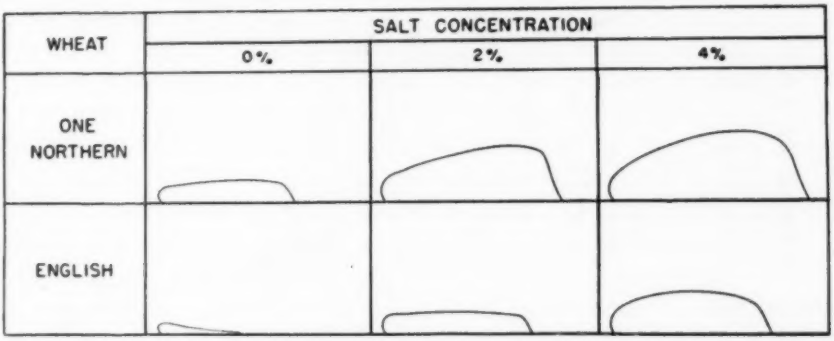

Fig. 5. Extensograms (45 min.) showing effects of varying the salt concentration in doughs.

baking, the selection seems to be arbitrary. No data on the effects of varying either factor have come to the authors' attention, and a study of the matter was therefore undertaken.

Experimental. Preliminary information on salt concentration was obtained from extensograms made with a strong Canadian flour (E) and a weak English flour (N). By the baker's tactile judgment, these two flours exhibited extremes in dough-handling properties. Consistency was adjusted to 540 units, a level which corresponds to that used in this laboratory for baking tests. Salt concentrations were 0, 2, and 4%, and doughs were mixed for 3 minutes in the Hobart mixer. Fig. 5 shows the extensograms made 45 minutes after mixing.

With both flours, increasing the salt concentration increases not only the resistance but also the extensibility. In the extensograms for the English flour the high point of the curve is near the beginning for no salt and moves towards the center as salt concentration is in-

creased. The shape of the extensogram for Canadian flour also changes with salt concentration by becoming progressively steeper. If extensibility is judged by the total length of the curve (E), the Canadian dough is nearly twice as extensible as the English with no salt; but with 2 and 4% of salt, differences in extensibility are considerably reduced. If extensibility is judged by the length of the curve to its highest point (E_1), the Canadian dough is more than 10 times as extensible as the English dough with 0 and 2% salt, and nearly three times as extensible with 4% salt. (Fig. 9 illustrates the dimensions E and E_1.)

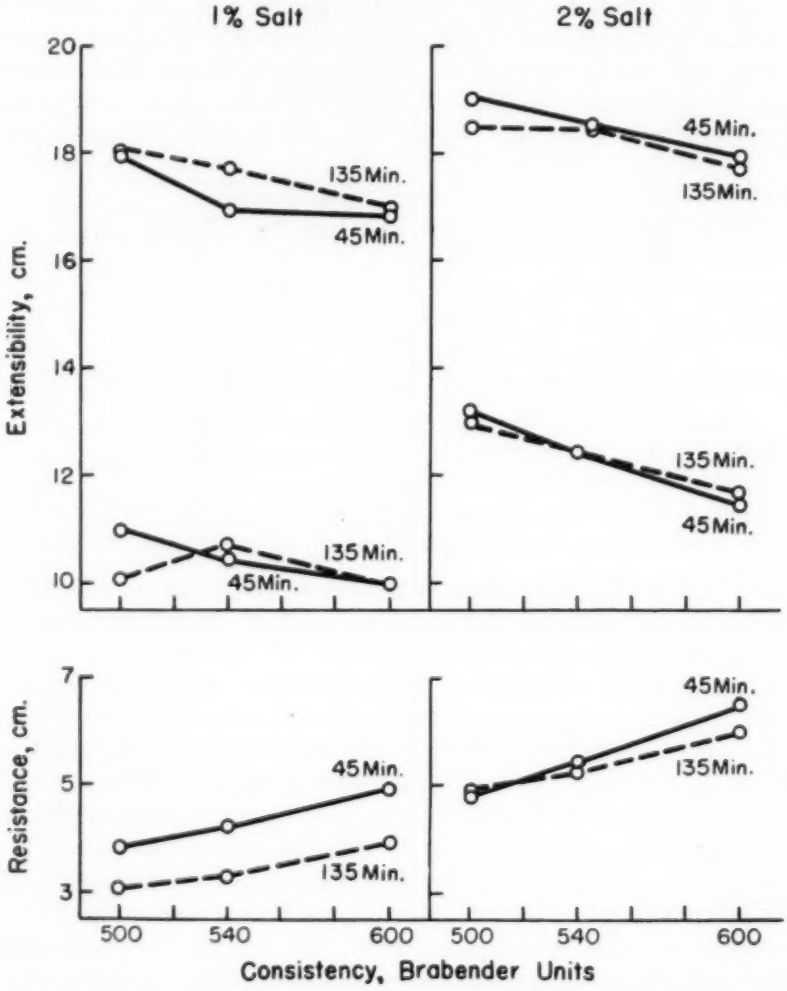

Fig. 6. Graphs showing effects on extensogram measurements of varying dough consistency and salt concentration. The data are mean values for flours E, F, H, I, O, and P. In top half of figure, upper lines represent total curve length (E) and lower lines represent curve length to maximum height (E_1).

In a larger study involving both salt concentration and consistency, extensograms were made from flours E, F, H, I, O, and P. Salt concentrations were 1% and 2%, and consistencies were 500, 540, and 600 units. The doughs were mixed for 3 minutes in a Hobart mixer, and extensograms were made 45 minutes and 135 minutes after mixing. The mean absorptions corresponding to these consistencies were 57.8, 56.8, and 55.1% with 1% of salt, and 58.5, 57.5, and 55.3% with 2% of salt.

Mean results are summarized for all flours in Fig. 6. Comparison of the data for the 45-minute extensograms (solid lines) with those for the 135-minute extensograms shows little difference between them. Since the mixing was mild, this was to be expected. Comparison of the curves on the left with those on the right shows that increasing the salt concentration increases extensibility and resistance. But the slopes of the curves show (with one exception) that increasing consistency decreases extensibility though it also increases resistance.

In the upper graphs, curves are given for both E (total curve length) and E_1 (length to greatest height). Both measurements react similarly to changes in salt concentration and consistency.

Data for 135-minute extensograms for four of the flours (E, P, F, and O) are plotted in Fig. 7. These curves are shown primarily to emphasize the differences between total extensibility (E) and extensibility to maximum height (E_1). Both with 1% of salt (top graphs) and with 2% of salt (bottom graphs) the spreads between curves for E and E_1 increase from flour E through flours P and F to flour O. Moreover, this generalization holds at all three levels of consistency. Accordingly, judgment of the comparative extensibilities of the flours will depend upon which measurement is made. For instance, the first and last flours are of about the same extensibility as judged by total extensogram length (E), but differ widely if judged by length to greatest height (E_1). Again, by the first measurement, flour O is more extensible than flour F, whereas O is less extensible if judged by the second measurement.

The 45-minute extensograms for these four flours are shown in Fig. 8 for consistencies of 500 and 600 units. Differences in the shapes of the extensograms are considerable. Average 1 Northern (E) yields curves with the highest point well towards the right, and the difference between the two extensibility measurements, E and E_1, is thus comparatively small. For the low protein 3 Northern (P), the point of maximum height varies in position with the amount of salt; with 1% salt it is towards the left, and with 2% it is towards the right. With both Amber Durum (F) and Ontario white winter (O) the point of maximum height is invariably towards the left, and with these flours

E and E_1 differ widely. On the basis of total extensibility (E), little difference exists between the top and bottom flours in Fig. 6; but on the basis of extensibility to maximum height (E_1), the top flour would be judged to be far superior. Accordingly, the significance of an extensogram cannot be defined merely in terms of length, height, and area; attention must also be paid to its shape, although this can probably

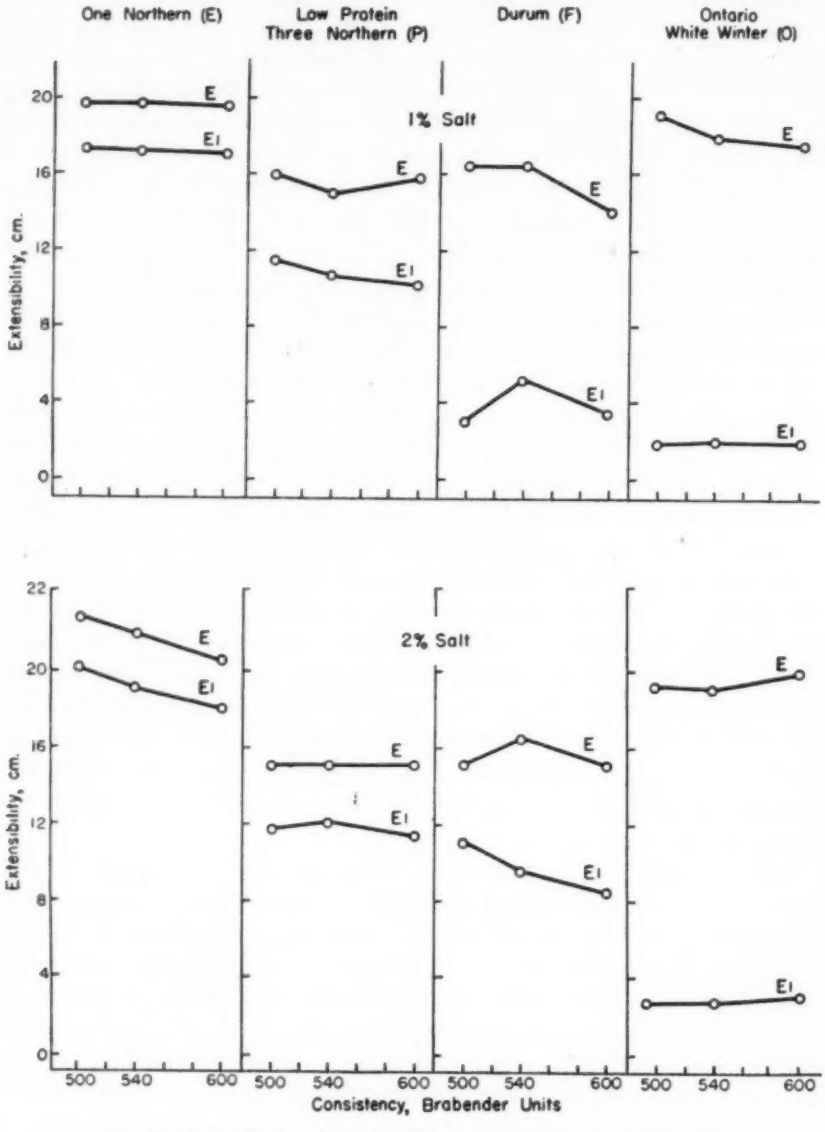

Fig. 7. Graphs for four flours showing effects on extensogram (135 min.) measurements of varying dough consistency and salt concentration.

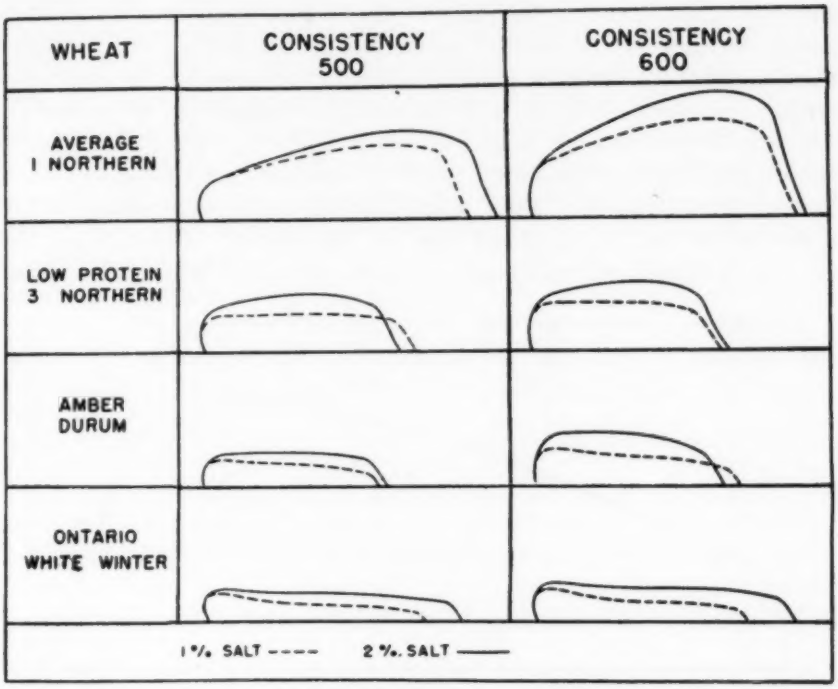

Fig. 8. Extensograms (45 min.) for four flours showing effects of different dough consistencies and salt concentrations.

be recorded for most purposes in terms of the measurements of E and E_1.

Discussion. The main question arising from these studies of salt concentration and consistency relates to the measurement of extensibility. Merritt and Bailey (4) have advocated that E_1 rather than E should be measured because the former is more reproducible. But it also appears that these two measurements represent different properties or combinations of properties of the flours. If this is true, a technique which magnifies differences between the two measurements may well prove useful.

The fundamental properties responsible for the behavior of a dough in the extensograph are elasticity and viscosity. Since both properties are involved throughout most of the stretching, the extensograph cannot be expected to provide independent measurements of each. Nevertheless, the shape of the extensogram does appear to indicate the relative importance of these two properties at various stages of stretching.

The mechanism of stretching must be somewhat as follows. Cohesive forces within the resting dough require that a certain stress be imposed before appreciable stretching can begin, and there is thus a

sharp immediate rise in the curve. An extension of the three-dimensional network of the fibrillar molecules of the gluten complex must then occur. As stretching is continued, the network of molecules within the gluten strands, and the strands themselves, are extended. The elastic limit is probably exceeded at various points distributed at random throughout the dough, and the cohesive forces between certain molecules are broken. This period appears to be represented in the extensogram by a continued rise in the curve. Throughout this process of elastic deformation, there must also be viscous deformation

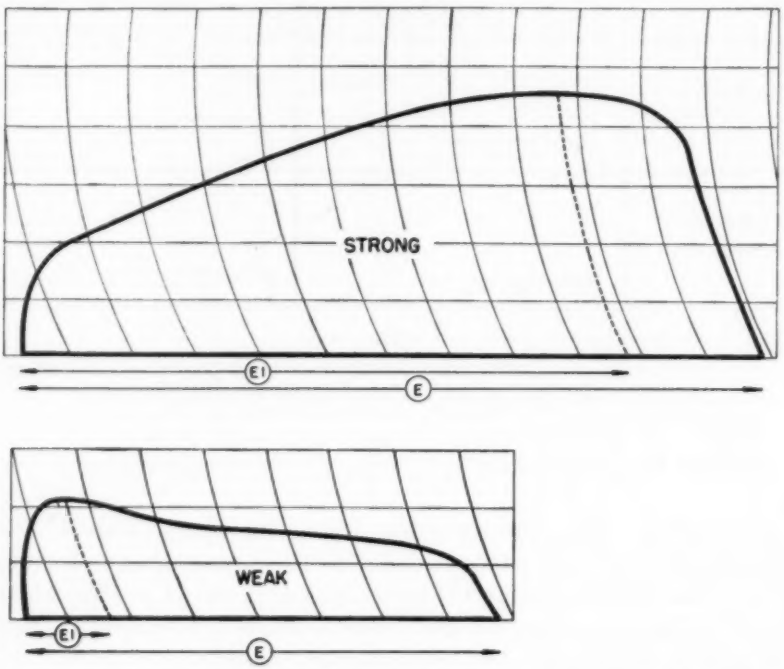

Fig. 9. Extensograms for strong and weak flours illustrating the measurement of E and E_1.

of the dough, which also affects the shape of the curve. When all elasticity has been overcome, plastic flow appears to continue and the curve tends to fall. Finally, the dough strand starts to tear as its tensile strength is exceeded and the curve begins to drop sharply. If strands on both sides of the hook tear at the same time, the drop in the curve is abrupt; but if one strand tears before the other, a more gradual drop occurs.

If dough had no elastic properties, the initial rise would be followed by a gradual decline in the curve representing the decreased resistance to viscous deformation as the strand became thinner with elongation. If the dough were entirely elastic, like a spring, a straight line, with

slope proportional to the stiffness of the spring, would be drawn. The extensogram represents a compromise between these two extremes.

This hypothetical account of the possible mechanisms of stretching leads to a speculative interpretation of the shape of extensograms. Representative curves for a strong and a weak flour are shown in Fig. 9. In both curves, the elastic properties are considered to offer resistance to stretching until the curve starts to drop, i.e., until the highest point is reached. The remaining part of the curve is considered to represent continued viscous deformation. Accordingly, the measurement E_1 probably reflects the elastic properties of the dough much more than the measurement E. The curve for the strong flour indicates a "bucky" dough, since some elasticity is retained throughout the stretching almost to the point at which breaking of the strands occurs. The curve for the weak flour indicates a dough that has little elasticity (E_1 is short) but is soft and reasonably extensible.

Results reported earlier in this section indicate that increasing the consistency of the dough makes the extensogram higher and shorter but has comparatively little effect on the relations between E and E_1. But increasing the salt concentration does appear to increase E_1 more than E and thus tends to mask differences in the elasticity of the flours. Increasing consistency, by reducing water content, probably affects the curve largely by increasing resistance to viscous deformation. But the salt appears to affect elasticity, presumably through action on the protein involving the Donnan effect.

In laboratories restricting studies to one type of flour, the levels of salt and consistencies selected for standard tests are probably of little consequence. All selections will place the flours in essentially the same rank order with respect to either measurement of extensibility. Indeed both measurements, E and E_1, will prove to be highly correlated. But, in laboratories dealing with a wide range of flour types, some advantage may be gained from selecting a technique that emphasizes the differences between E and E_1. For among different classes of flour, ranging from strong to weak, these two measurements are not correlated.

General Discussion

As a result of these studies, a basic technique has been adopted for extensograph tests made in this laboratory. It corresponds in usefulness to the basic baking formula. Thus it provides some information about the comparative qualities of different flours, but also serves as a control procedure for the comparison of the effects of changing formula and technique in various ways.

Salt cannot be omitted from the formula because many of the doughs are too sticky for convenient manipulation during the test.

But salt can be reduced to 1%, which is the level used in baking tests and produces manageable doughs. An absorption identical with that used for baking is the most logical selection, and this corresponds in this laboratory to a consistency of 540 farinograph units. Since the basic test is not required to demonstrate the differential response of flours to mixing, a mild mixing procedure was selected. This is again identical with that used in the baking test, namely, 3 minutes in the Hobart mixer. Tests made with a wide range of flours have shown that the rest period of 45 minutes recommended by the Brabender Corporation is adequate for the relaxation of doughs. Accordingly, a 150-g. dough is scaled after mixing, is then rounded and rolled with the extensograph equipment, and is stretched after a 45-minute rest in a cabinet maintained at 86°F. and 85% relative humidity.

This technique is used mainly for comparative studies of varieties, especially for testing new lines and selections produced by plant breeders. When material is limited, useful results can also be obtained with a 75-g. dough without varying the technique in any other way. Curves are smaller, but their measurements are comparable to those of curves for 150-g. doughs.

Provided that procedure and formula do not vary widely from those described above, there will be little difference between the curves drawn 45 minutes after mixing. As a broad generalization, it may thus be said that all 45-minute curves represent essentially the innate properties of the flour. To measure the effect of treatments such as severe mixing or bromation, a second curve must be drawn. In this laboratory the second curve is prepared 135 or 180 minutes after mixing, with the proviso that the dough be rounded and rolled 45 minutes before stretching. The difference between the first curve (45 minutes) and the second curve (135 or 180 minutes) is considered to represent the response of the flour to treatment; for if the treatment were not varied from that of the basic procedure, the first and second curves would be almost identical. Perhaps there is here involved a mere question of terminology; and "innate properties" and "response" may not be the best terms available. Nevertheless, these terms do appear to convey a useful concept of what the extensograph measures and of how the results should be interpreted.

The principal limitation of the extensograph is that it does not measure specific physical properties such as elasticity and viscosity. The machine stretches a dough at constant rate and measures extension and resistance to extension. Though these properties depend upon the elasticity and viscosity of the dough, they are recorded in units that cannot be interpreted clearly in terms of either. The units have no independent meaning. They can be defined only in terms of the

machine, of its adjustment, and of the technique used in making the test. Accordingly, the units are wholly empirical, and this in itself sets a limit to the usefulness of the machine and of others like it. Ideal measurements of the rheological properties should yield fundamental constants such as elastic moduli and viscosity coefficients and should not depend on the machine used. Among dough studies of this type, those of Halton and Scott Blair (2, and papers cited therein) are preeminent. But their equipment and technique are hardly suitable for routine studies. Accordingly, the Brabender Extensograph, which is extremely convenient and simple to operate, will probably continue to be widely used in spite of its limitations.

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INACTIVATION AND REMOVAL OF PROTEOLYTIC ENZYMES FROM AMYLOLYTIC SUPPLEMENTS [1]

B. M. DIRKS [2] and BYRON S. MILLER [3]

ABSTRACT

Silicates proved superior to several other commercial adsorbents for selectively removing proteolytic enzymes from amylolytic supplements.

It was observed that 50 to 60% of the proteolytic activity in a fungal extract was inactivated rapidly and irreversibly by adjusting the pH to 10.0, with little decrease in amylolytic activity.

The addition of sodium chloride (4.3 *M*) to a fungal extract caused marked reduction of proteolytic activity and relatively little loss of amylolytic activity. Dialysis against 0.2% calcium chloride solution for 36 hours did not reactivate the proteolytic enzymes. In the presence of sodium chloride (4.3 *M*), adjustment of the extract to pH 10.0 resulted in 80 to 90% reduction of the original proteolytic activity with a small, but significant, loss of alpha-amylase.

Similar effects were noted in extracts of malted wheat flour and malted barley. The proteolytic activity of bacterial amylolytic preparations, however, was only slightly affected by increasing the reaction to pH 10.0. High concentrations of sodium chloride inactivated relatively large amounts of bacterial amylolytic, as well as proteolytic, enzymes.

Supplementation of flour with excessive amounts of alpha-amylase preparations has proved harmful in many instances. The reasons for these detrimental effects have been the subject of much research. Miller and Johnson (7) reviewed the literature dealing with this problem and presented data supporting the premise that the effects are due primarily to high proteolytic activity present in amylolytic supplements. Since controlled proteolytic activity is important in brewing and distilling processes as well as breadmaking, a study of methods for control of this enzyme activity in amylolytic preparations appears warranted.

Several general procedures have been used for purification and concentration of enzymes. These include dialysis, fractional precipitation, selective adsorption followed by elution, electrophoretic separation, and differential inactivation. The use of adsorbents has produced

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A portion of a thesis presented by B. M. Dirks as partial fulfillment of the requirements for the degree of Master of Science in Milling Industry at Kansas State College.
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promising results, but the problem is complex and involves the interaction of many factors. The reaction or pH of the adsorbent suspension has a marked effect on the specificity and efficiency of the adsorption process. Lüers and Malsch (5) and Sabalitschka and Weidlich (8) found that malt amylase was best adsorbed at pH values approximating 4.5, while a pH optimum above 5.0 was observed for the adsorption of malt proteases. A series of investigations by Kitano (2, 3, 4) showed that the selectivity of any particular adsorbent for amylase or maltase was not absolute, but was influenced by temperature, pH, and enzyme concentration.

In 1938, Waldschmidt-Leitz and Ziegler (10) obtained a patent on the use of bauxite, a natural aluminum ore, for selectively adsorbing proteases from enzyme mixtures obtained from malted grains, molds, and bacteria. This process succeeded only in increasing the ratio of amylolytic to proteolytic enzymes. A pH value between 5.5 and 7.5 was recommended. Miller and Johnson (7) have investigated kaolin and found this agent also to be effective in differentially removing proteolytic enzymes although the separation was not complete. The purpose of the present work was to investigate various adsorbents and associated treatments in an effort to develop methods for the inactivation or removal of proteolytic from amylolytic enzymes.

Materials and Methods

The chief source of amylase investigated was that from the mold *Aspergillus oryzae*, cultured on wheat bran. Bacterial and cereal amylase preparations, as well as other fungal sources, were also used for comparative purposes. A solution of amylolytic and proteolytic enzymes was prepared by extracting the source with 0.2% calcium chloride solution at room temperature. A ratio of 1 g. of preparation to 10 ml. of 0.2% calcium chloride solution provided an extract of convenient enzyme activity. The mixture was stirred every 15 minutes for one hour after which it was filtered and centrifuged.

Commercial adsorbents used in this investigation were Duolite A-2, Duolite C-3, Florisil, Florex XXX, Isco-Adsorbol N-100, Isco-Adsorbol A-420, Santocel C, Santocel CX, Deacidite, Decalso Fines, Folin Decalso, Zeo-Dur, Zeo-Karb H, Zeo-Rex H, Amberlite IR4B, Amberlite IR100, and Zeolite. Other materials tested included Norit A, a decolorizing carbon of the Pfanstiehl Chemical Co., Waukegan, Ill.; Lloyd's Reagent, Hartmann-Leddon Co., Philadelphia; activated alumina; egg albumin; pumice; permutite; and Turkish emery.

Several additional adsorbents were prepared in the laboratory as follows:

Activated bauxite—

A sample of pisolitic gray massive bauxite ore from Arkansas was finely ground and activated at 200°C. for 24 hours.

Hydrous aluminum oxide gel—

Prepared by adding 250 ml. of 3 *M* ammonium hydroxide solution to 250 ml. 0.5 *M* aluminum sulfate solution. The precipitate was washed, dried at 130°C., and activated for 24 hours at 200°C.

Hydrous aluminum oxide gel on asbestos support—

Prepared in the same manner as above but in the presence of 10 g. asbestos fiber.

Hydrous aluminum oxide gel in alcohol—

Prepared in the same manner as above with the alcohol being added at the time of adsorption.

Magnesium silicate—

Made by diluting sodium silicate to a specific gravity of 1.06 with distilled water and adding a molar solution of magnesium chloride to the silicate as long as precipitation occurred. The precipitate was washed, dried at 130°C., and activated 24 hours at 200°C.

Adsorption was carried out in a batch process at room temperature. A 25-ml. portion of the enzyme extract diluted 1:10 was added to 2 g. of the adsorbent in a 50-ml. beaker and stirred periodically during a 30-minute period. The pH of the suspension was measured immediately after mixing and pH adjustments were made at this time unless otherwise noted. After standing, the suspension was filtered with suction through Whatman No. 5 filter paper. Alpha-amylolytic activity and proteolytic activity were determined on appropriately diluted aliquots of the filtrate. All dilutions were made with 0.2% calcium chloride solution. This concentration (0.018 *M*) of calcium chloride did not inhibit proteolytic activity.

Proteolytic activity was determined by a modified Ayre-Anderson procedure as standardized by Miller (6). Briefly, this method involves the hydrolysis of a hemoglobin substrate by the enzyme extract in a mixture buffered at pH 4.7. After the hydrolysis period, undigested protein was precipitated with trichloroacetic acid, filtered off, and aliquots of the filtrate analyzed for total nitrogen by the Kjeldahl process. Titration values in ml. 0.0714 *N* sodium hydroxide indicated the proteolytic activity in the sample.

Alpha-amylase activity was determined by the starch dextrinization procedure described by Sandstedt, Kneen, and Blish (9). Values for alpha-amylase activity were expressed as the time required for the

digestion mixture to produce a standard red-brown end point with iodine.

Results and Discussion

Enzyme Concentration-Activity Relationships. Sandstedt, Kneen, and Blish (9) have established the linear relationship between alpha-amylase concentration and the rate of dextrinization, but no such simple relationship was evident in the determination of proteolytic activity. It was therefore necessary to determine the relation between proteolytic enzyme concentration and the titration values obtained from the activity determination. The activity expressed in ml. 0.0714 *N* sodium hydroxide (blank minus sample titrations) was plotted as shown in Fig. 1.

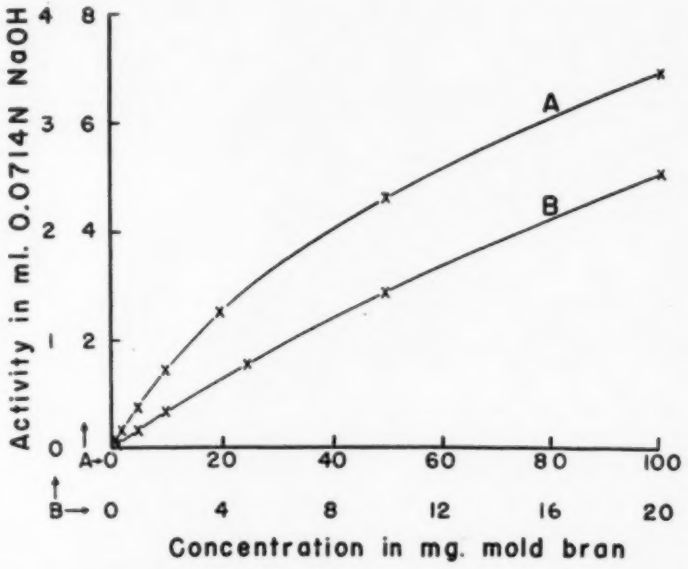

Fig. 1. Effect of concentration on measured proteolytic activity of mold bran extracts. Curve B represents the first portion of Curve A plotted on a different scale.

Curve A demonstrates the lack of proportionality between activity and enzyme concentration. Titration values consequently must be referred to a previously determined curve to get an accurate estimate of proteolytic activity. In this investigation it was convenient to dilute extracts so that titration values did not exceed 3 ml. These values fall on the portion of the curve plotted in curve B.

Examination of Adsorbents. Preliminary work was designed to eliminate those adsorbents showing little or no faculty for selective adsorption of proteolytic enzymes. Each adsorbent was tested in duplicate and the average results are given in Table I. These data

indicate that the use of Santocel C, Santocel CX, Zeo-Karb H, Zeo-Rex H, Isco-Adsorbol A-420, Duolite A-2, and Duolite C-3 resulted in pH levels that inactivated both proteolytic and amylolytic enzymes. These adsorbents should not be eliminated conclusively since preliminary treatment could adjust the pH to almost any level desired with probable improvement in selective properties. Other adsorbents including Florex XXX, Norit A, Zeo-Dur, egg albumin, and aluminum

TABLE I

EFFECTIVENESS OF VARIOUS ADSORBENTS IN SELECTIVE ADSORPTION OF PROTEOLYTIC AND AMYLOLYTIC ENZYMES

Adsorbent	pH	Per cent loss in activity	
		Amylolytic	Proteolytic
Amberlite IR 4B	10.8	61.1	85.6
Amberlite IR 100	10.2	30.0	88.2
Florex XXX	6.5	92.7	97.6
Florisil	9.2	66.7	100.0
Santocel C	3.3	83.7	81.1
Santocel CX	3.5	85.0	80.4
Folin Decalso	6.3	7.2	33.8
Decalso Fines	7.2	7.2	61.8
Zeo-Dur	6.1	10.4	13.5
Zeo-Karb H	1.7	100.0	94.8
Zeo-Rex H	1.3	100.0	93.6
De-Acidite	7.6	29.0	51.8
Activated alumina	7.5	53.4	77.2
Magnesium silicate	10.2	22.8	78.9
Turkish emery	5.5	3.5	17.0
Pumice (pwd.)	5.8	13.0	44.4
Egg albumin	6.2	10.0	20.5
Isco-Adsorbol A-420	3.4	100.0	100.0
Isco-Adsorbol N-100	6.4	29.0	84.7
$Al(OH)_3$	6.5	44.0	31.1
$Al(OH)_3$ on asbestos	6.5	42.8	52.8
$Al(OH)_3$ in alcohol	4.3	19.4	22.8
Activated bauxite	6.2	22.2	40.5
Zeolite	4.7	17.6	51.8
Norit A	7.2	100.0	97.1
Permutite	6.9	6.6	31.5
Duolite A-2	3.6	100.0	53.5
Duolite C-3	2.1	100.0	96.5
Lloyd's Reagent	6.7	12.1	78.0

hydroxide exhibited little selectivity in their adsorption characteristics under the conditions of these experiments.

The remaining adsorbents were subjected to further study by varying the pH through the addition of dilute hydrochloric acid or sodium hydroxide to the suspension immediately after mixing the enzyme extract and adsorbent. Figs. 2 and 3 show the results obtained with the eight adsorbents exhibiting the greatest relative preference for proteolytic enzymes.

Percentage losses of proteolytic and amylolytic activity are plotted against various pH levels for each adsorbent. Points represent average values of several determinations within a pH range of 0.5 unit. The degree of adsorptive selectivity for the adsorbent concentrations used is thus represented by the distance between the two curves at any particular pH value. Since higher values invariably resulted in increased losses of amylolytic activity, the curves were not extended far above pH 10.0. Adsorption treatment of extracts previously

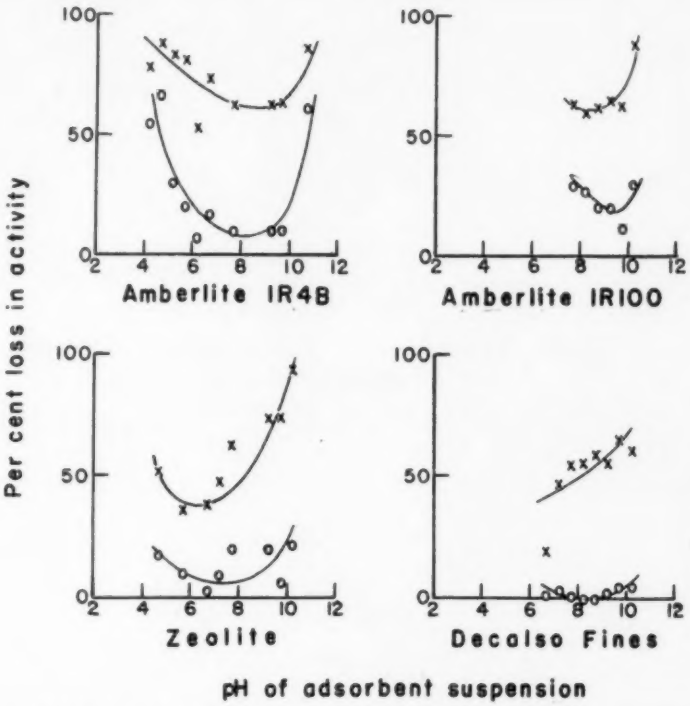

Fig. 2. Relationship of adsorption of amylolytic and proteolytic enzymes in mold bran extracts to changes in hydrogen-ion concentration for different adsorbents. Amylolytic activity is represented by "o" and proteolytic activity by "x."

adjusted to pH 10.0 decreased the proteolytic activity over 80% at the expense, however, of significant losses in amylolytic activity.

Removal of proteolytic enzymes from amylolytic supplements by adsorption was only partially successful. The adsorbents exhibiting the best selective properties were either silicates or were high in silicate content. Best results were obtained with Decalso, a synthetic sodium alumino-silicate; Lloyd's reagent, a form of hydrated aluminum silicate; and Isco-Adsorbol N-100, a natural clay. Additional work employing various quantities of specific adsorbents was abandoned

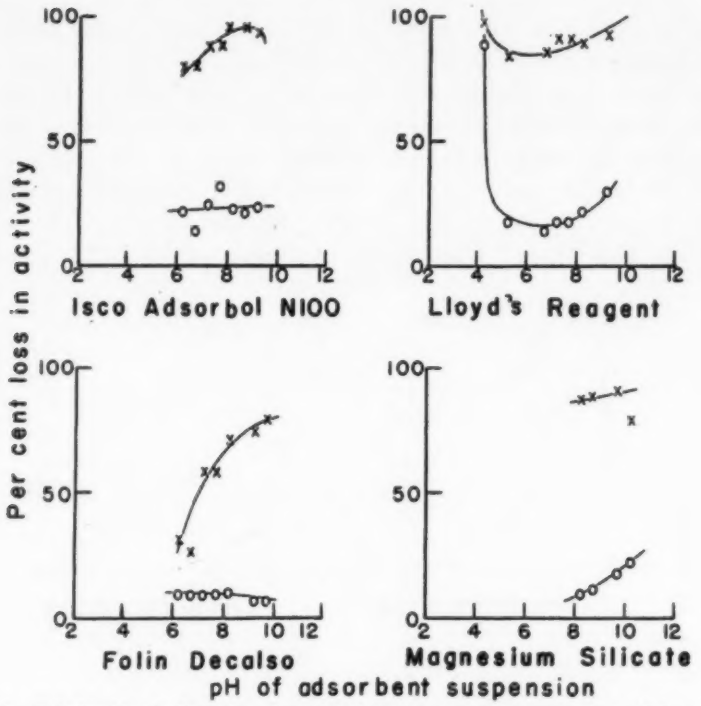

Fig. 3. Relationship of adsorption of amylolytic and proteolytic enzymes in mold bran extracts to changes in hydrogen-ion concentration for different adsorbents. Amylolytic activity is represented by "o" and proteolytic activity by "x."

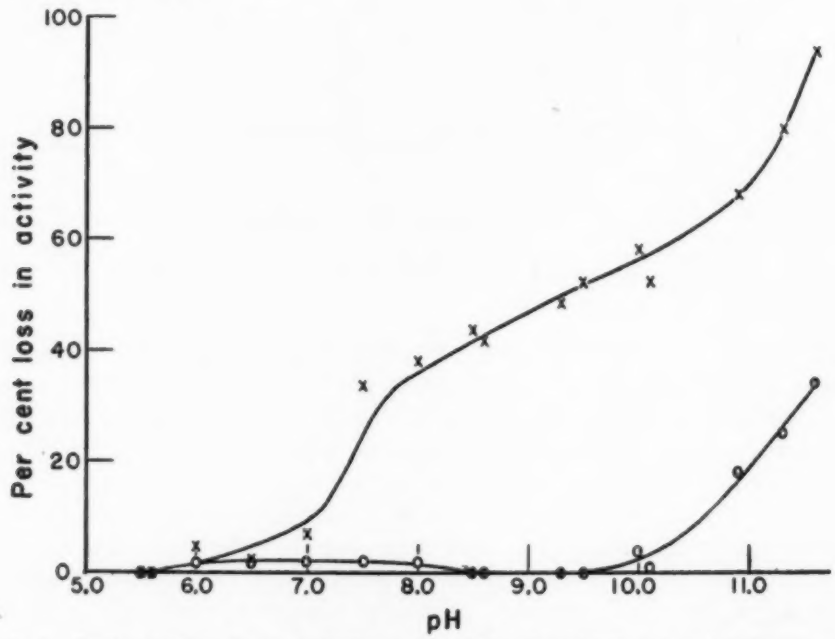

Fig. 4. Effect of hydrogen-ion concentration on the activity of amylolytic and proteolytic enzymes in mold bran extracts. Amylolytic activity is represented by "o" and proteolytic activity by "x."

due to the more promising results obtained by controlled hydrogen-ion and chloride-ion concentration.

Effect of Hydrogen-Ion Concentration. The curves in Figs. 2 and 3 show that the highest degree of adsorptive selectivity apparently occurred between pH 8.0 and pH 10.0 in nearly all cases. This fact stimulated further investigation of pH effects. The data plotted in Fig. 4 show that the effect of high pH values was largely due to selective inactivation rather than to adsorption. A pH of 10.0 resulted in the inactivation of over 50% of the proteolytic activity with no significant loss in amylolytic activity. Thus there appeared to be no advantage in the use of adsorbents for removal of proteolytic enzymes unless a selective adsorption of at least 50% over the amylolytic enzymes was obtained.

TABLE II

EFFECT OF HYDROGEN-ION ADJUSTMENT WITH DIFFERENT REAGENTS ON AMYLOLYTIC AND PROTEOLYTIC ACTIVITY OF MOLD BRAN EXTRACTS

Treatment	pH of extract	Per cent loss in activity	
		Amylolytic	Proteolytic
NaOH	10.1	1.9	51.7
$Ba(OH)_2$	10.0	5.9	45.5
NaOH+HCl	5.6	12.7	61.4
NaOH+HAc	4.6	5.8	53.3

Table II reveals that adjustment of pH with dilute barium hydroxide achieved the same degree of inactivation as adjustment with dilute sodium hydroxide. The resulting proteolytic activity after 3 hours was virtually the same as when measured immediately after pH adjustment. Further the proteolytic enzymes did not recover their activity either with standing as long as 36 hours or with subsequent lowering of the pH level. The inactivation, apparently due to hydroxyl ions, was concluded to be irreversible and very rapid. Readjustment of pH by the addition of dilute hydrochloric acid caused an additional loss of proteolytic activity. Since acetic acid caused no significant changes, it was assumed that the additional loss of proteolytic activity with the addition of hydrochloric acid was due to the chloride ion.

Effect of Sodium Chloride. Table III shows the effect of sodium chloride on enzyme activity when the salt was added to the hemoglobin digestion mixture, the enzyme extract, and the extraction slurry. A salt concentration equivalent to 4.3 *M* in the extract reduced the proteolytic activity about 20 to 30% with little loss of amylolytic activity irrespective of the time of addition. As noted previously, the

TABLE III

EFFECT OF SODIUM CHLORIDE ON AMYLOLYTIC AND PROTEOLYTIC ACTIVITY OF MOLD BRAN EXTRACTS

Treatment	NaCl molarity	pH of extract	Per cent loss in activity	
			Amylolytic	Proteolytic
In digest	0.6[1]	5.2	14.8	66.4
In digest	0.2[2]	5.2	2.0	26.2
In extract	0.5	5.2	0.0	3.3
In extract	4.3	4.8	5.8	25.2
In slurry	4.3	5.4	3.8	17.6

[1] 0.6 *M* sodium chloride in the digestion mixture represents the same concentration as that usually mixed in bread doughs.
[2] 0.2 *M* sodium chloride in the digestion mixture is equivalent to 4.3 *M* sodium chloride in the enzyme extract.

original mold bran source was extracted with 0.2% calcium chloride solution. This represents only 0.036 *M* chloride ion, and at least 0.5 *M* chloride ion was necessary before any appreciable loss of proteolytic activity was detected. Since concentrations of calcium chloride supplying equivalent amounts of chloride ion achieved results similar to those obtained with sodium chloride, the results were apparently due to the chloride ion present.

Dialysis of treated extracts for 36 hours at 30°C. against 0.2% calcium chloride solution resulted in no change in activity. Quantitative determinations of chloride ions showed the chloride concentration to be reduced to 0.036 *M* during this period. Treated and untreated check samples allowed to stand for the same length of time at 30°C. exhibited no change in activity from that measured immediately after treatment. The inactivating influence of chloride ions consequently appears to be quite rapid and irreversible.

Combined Effect of Hydrogen-ion Concentration and Sodium Chloride. The additive effect of hydroxyl and chloride ions on proteolytic

TABLE IV

COMBINED EFFECT OF SODIUM CHLORIDE AND HYDROGEN-ION CONCENTRATION ON THE AMYLOLYTIC AND PROTEOLYTIC ACTIVITY OF MOLD BRAN EXTRACTS

Treatment	NaCl molarity	pH of extract	Per cent loss in activity	
			Amylolytic	Proteolytic
NaOH	—	10.1	1.9	51.7
NaCl	4.3	4.8	5.8	25.2
NaCl+NaOH	4.3	10.1	6.5	86.2
NaCl+NaOH (in slurry)	4.3	6.9[1]	10.7	95.1

[1] The slurry was adjusted to pH 10.0 before centrifuging and diluting for analysis.

activity is shown in Table IV. Treatment of mold bran extracts at pH 10.0 with 250 mg. sodium chloride per ml. resulted in over 80% inactivation of proteolytic enzymes and less than 10% loss of amylolytic enzymes. Significant losses of amylolytic activity prevented the use of higher concentrations of either hydroxyl or chloride ions. Additional work concerning the effect of other ions is in progress.

Comparison of Enzyme Sources. Table V shows the effects of hydroxyl and chloride ions on the enzyme activity of amylase preparations from various sources. Proteolytic and amylolytic enzymes from several fungal sources reacted similarly to treatment with sodium chloride and sodium hydroxide. The cereal and vegetable enzymes also were inactivated in the same manner, although the proteolytic

TABLE V

EFFECT OF HYDROGEN-ION CONCENTRATION AND SODIUM CHLORIDE ON THE AMYLOLYTIC AND PROTEOLYTIC ACTIVITY IN EXTRACTS OF DIFFERENT ENZYME SOURCES

Source	Type	Per cent loss in activity					
		pH 10.0		0.6 *M* NaCl[1]		pH 10.0, 0.6 *M* NaCl	
		Amyl.	Prot.	Amyl.	Prot.	Amyl.	Prot.
Goering bran	Fungal	0.0	52.0	14.8	66.4	14.8	88.0
Wallerstein bran	Fungal	4.8	76.5	9.1	54.1	16.7	88.8
E-Z-1	Fungal	0.0	75.0	10.5	48.3	10.5	86.7
Rhozyme S	Fungal	0.0	85.7	5.9	45.7	5.9	92.1
Wallerstein	Bacterial	0.0	1.1	10.5	40.4	10.5	40.4
Jeffrey's bran	Bacterial	3.8	3.9	35.1	71.0	35.9	80.6
Polidase-S	Vegetable	0.0	30.5	0.0	56.6	5.3	78.6
Malted barley	Cereal	0.0	70.6	2.4	26.5	4.7	78.4
Malted wheat	Cereal	0.0	30.4	6.7	15.8	12.5	42.9

[1] 0.6 *M* sodium chloride in the digestion mixture represents the same concentration as that usually mixed in bread doughs.

activity of malted wheat flour was not decreased to the same extent as in malted barley and fungal extracts. For all fungal and cereal sources tested, there was a definite additive effect of pH and salt inactivation resulting in as much as 90% loss of proteolytic activity with as little as 6% loss of amylolytic activity.

Bacterial sources, however, provided proteolytic enzymes with different properties. Proteolytic activity was not decreased significantly at pH 10.0, but amylolytic activity decreased sharply with pH values above 10.0 in the same manner as the fungal amylolytic activity. The same inactivation pH for bacterial amylases was reported by Di Carlo and Redfern (1). Treatment with sodium chloride achieved a degree of proteolytic enzyme inactivation comparable to that of fungal sources. With higher salt concentrations it was possible to

obtain an inactivation of bacterial proteolytic activity roughly equivalent to that obtained with fungal extracts treated with both salt and base. However, a relatively large amount of amylolytic activity also was lost.

The inactivation or removal of proteolytic enzymes from amylolytic supplements was not complete, but a significant increase in the ratio of amylolytic to proteolytic activity was successfully obtained. However, only a few of the many aspects of the problem have been covered.

The use of adsorption and elution techniques should be exploited to determine proper conditions for this separation. Investigations of the amylolytic and proteolytic enzyme systems of cereals, molds, and bacteria would then be facilitated.

Acknowledgments

Acknowledgment is made to the several commercial concerns who generously supplied the adsorbents and enzyme preparations used in this investigation. The mold bran used as the chief source of amylase studied was obtained through the courtesy of Dr. K. J. Goering, President, the Mold Bran Company, Eagle Grove, Iowa. Additional fungal sources included "E-Z-1" from the Mold Bran Company; a mold bran product of the Wallerstein Laboratories, New York; and Rhozyme-S, Rohm and Haas Company, Philadelphia. A bacterial bran of the Jeffreys Laboratories, Salem, Virginia, was used as well as a Wallerstein bacterial amylase preparation. Also examined were a commercial malted wheat flour, the Kansas Milling Company, Wichita, Kansas; a commercial malted barley, Hales and Hunter Company, Chicago; and Polidase-S, a vegetable amylase preparation of the Schwartz Laboratories, Inc., New York.

Samples of adsorbents were obtained from the following sources:

The Chemical Process Co., 58 Sutter Street, San Francisco—Duolite A-2, Duolite C-3.

The Floridin Co., Warren, Pennsylvania—Florisil, Florex XXX.

Innis, Spieden and Co., 117 Liberty St., New York—Isco-Adsorbol N-100, Isco-Adsorbol A-420.

Monsanto Chemical Co., Merrimac Div., Boston—Santocel C, Santocel CX.

The Permutit Co., 330 West 42nd St., New York—Deacidite, Decalso Fines, Folin Decalso, Zeo-Dur, Zeo-Karb H, Zeo-Rex H.

The Resinous Products and Chemical Co., Washington Square, Philadelphia—Amberlite IR4B, Amberlite IR100.

Zeolite Chemical Co., 140 Cedar St., New York—Zeolite.

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GRAIN STORAGE STUDIES. VIII. RELATION OF MOLDS IN MOIST STORED COTTONSEED TO INCREASED PRODUCTION OF CARBON DIOXIDE, FATTY ACIDS, AND HEAT [1]

CLYDE M. CHRISTENSEN,[2] JON H. OLAFSON,[3] and W. F. GEDDES[4]

ABSTRACT

Storage molds increased rapidly on and within cottonseed stored at relative humidities of 84% to 90% for 10 days at 30°C. In general, the increase in mold population of the stored seeds was correlated closely with increased production of carbon dioxide and free fatty acids. Because of the peculiar structure of the seed coat, molds gain easy access to the interior of moist stored cottonseed where they grow vigorously and sporulate profusely, so that cottonseed may be extremely moldy without betraying any external evidence. Fungicides applied to the outside of the seed did not prevent rapid growth and sporulation of molds within the seed coat. Entire meats and hulls stored moist without a fungicide respired more rapidly, had a higher fatty acid content, and bore a larger mold population than similar fractions treated with thiourea. The evidence suggests that storage molds may play an important part in the deterioration of moist stored cottonseed.

During the past 20 years considerable evidence has accumulated to indicate that molds often are a major factor in the spoilage and heating of moist stored seeds of various kinds (8, 16–23). Whether molds are similarly involved in the deterioration of moist stored cottonseed has been questioned, although cottonseed is known to bear a fairly rich internal flora of both parasitic and saprophytic molds (3). Malowan (15) found that the respiration of moist cottonseed was not reduced when the seed was treated with copper sulfate or mercuric chloride,

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and concluded that therefore microorganisms were not involved in its respiration. He did not determine whether the mold population of the seeds increased or decreased during his tests, and therefore had no experimental basis for supposing that the seed treatments he used eliminated molds. Darsie *et al.* (6), some years before, had found that treatment of seeds with copper sulfate did not prevent subsequent molding and heating of the seeds, and mercuric chloride was not always completely effective in eliminating molds. That fungicides applied to seeds may not inhibit the subsequent development of storage molds on the seed was shown by Milner, Christensen, and Geddes (17). In the absence of direct supporting evidence, therefore, Malowan's conclusion that microflora were not concerned with respiration of the seeds in his tests must be regarded as purely hypothetical. More recently, workers at the Southern Regional Research Laboratory have expressed some doubt as to whether molds are involved in the deterioration of moist stored cottonseed (1, 11, 12, 14). They favor the view that the major lipolytic, respiratory, and heating activity of cottonseed in storage is due to seed enzymes and that microbiological activity is, at best, a secondary factor which only comes into play after long storage. However, they did not determine the mold population in their experiments; and whether molds are involved in the deterioration of moist stored cottonseed, as they have been proven to be with other kinds of seed, remains a moot question. The present work was undertaken to explore some aspects of this problem.

Materials and Methods

The cottonseed used in these tests was, unless otherwise stated, nondelinted seed of the variety Stoneville, obtained from the Southern Regional Research Laboratory at New Orleans, Louisiana. In the various tests it was conditioned to the desired moisture contents by adding distilled water, then storing the seed at 2°–4°C. for 36 to 48 hours to allow the water to become distributed. Moisture content was determined by the method specified for cottonseed by the American Oil Chemists' Society (2). Carbon dioxide production was measured by the technique described by Milner and Geddes (18); the experimental lots of seed at various moisture contents were kept in respirometer bottles at 30°C., and air in hygroscopic equilibrium with the initial moisture content of the seed was continuously passed through the seed. At intervals of 24 hours the accumulated air was analyzed for carbon dioxide and oxygen, using a modified Haldane-Henderson gas analyzer.

Fat acidity was measured by a slight modification of the A.O.C.S. method for cottonseed (2). The intact seed was delinted with con-

centrated sulfuric acid, thoroughly washed in a stream of water, and air dried. The material was ground in an intermediate Wiley mill equipped with a 20 mesh screen, Samples of 20 g. were extracted at room temperature by allowing 100 ml. of petroleum ether to percolate through the material in a Butt-type extraction tube. The extracts were collected in weighed flasks and were reweighed, after evaporation of the solvent, to give the weight of oil present in the titration mixture. Subsequent treatment followed that recommended by the American Oil Chemists' Society.

The mold population was determined by grinding the seed in a small attrition mill turned by hand or in a Wiley mill equipped with a 20 mesh screen, and culturing the resultant meal in malt-salt agar as described by Christensen (4). It was difficult to reduce these oily seeds to a fine meal in the attrition mill, but eventually it was found that if the seeds were dried thoroughly, then chilled or frozen, they could be ground satisfactorily in the Wiley mill. Because of the unsatisfactory milling of some seed lots, it is likely that mold counts on these lots were lower than the actual population, but this probably had relatively little effect on the general results. To determine the percentage of seeds in which internal molds were present, 50 seeds were delinted in concentrated sulfuric acid, washed in running tap water, then surface-disinfected by washing in a 1.5% solution of sodium hypochlorite for 2 minutes (to eliminate any contaminants acquired from the tap water or from the air), placed on agar in petri dishes, and notes taken after 5 to 7 days.

The percentage germination of the seed was determined by the Minnesota State Seed Testing Laboratory.

Results

Relation of Moisture Content to Respiration, Mold Population, and Increase in Fatty Acids. Portions of seed weighing from 250 to 600 g. were conditioned to six different moisture contents, placed in respirometer bottles, and aspirated with air in hygroscopic equilibrium with each moisture content. In no case did the final moisture content differ from the initial by more than 0.5%. After storage for 10 days, the seeds were removed, dried, and the fat acidity, germination, total mold population, and percentage of seed with internal molds determined. The results are presented in Table I. The increasing internal infection by storage molds with increasing moisture content is illustrated in Fig. 1.

Respiration, fat acidity, and mold population increased slightly during the 10-day storage period at 80% relative humidity, and increased greatly at relative humidities of 84 to 90%. At 90% relative

TABLE I

CARBON DIOXIDE PRODUCTION, FAT ACIDITY, AND MOLD POPULATION IN COTTONSEED STORED AT SIX DIFFERENT RELATIVE HUMIDITIES, AT 30°C., FOR 10 DAYS

Number	Relative humidity	Moisture content	Respiration[1]	Fat acidity		Germination	Molds		Kinds of molds
				As oleic	Acid value[2]		Total number[3]	Internal infection[4]	
	%	%	*mg.*	%	*mg.*	%		%	
Original seed	—	—	—	0.4	8.1	97	700	6	*Fusarium vasinfectum*
1	65.0	9.6	5.6	0.5	10.4	93	0	8	*Fusarium vasinfectum*
2	75.0	11.6	7.0	0.6	11.9	88	700	0	Not identified
3	80.0	13.4	21.1	0.9	16.8	91	1,000	6	*Aspergillus glaucus*
4	84.0	15.2	79.0	1.3	26.0	90	3,300	24	*A. glaucus* and *A. versicolor*
5	87.0	17.4	406.2	6.3	125.1	66	152,000	24	*A. glaucus* and *A. versicolor*
6	90.0	19.8	1069.2	9.4	186.6	55	506,000	66	*A. glaucus* and *Penicillium* sp.

[1] Total mg. CO_2/100 g. dry matter in 10 days.
[2] Expressed as mg. KOH/10 g. of oil.
[3] Molds per gram.
[4] Percentage of seeds with internal molds.

humidity, the total carbon dioxide production was over twice as great as at 87%, free fatty acids about one and one-half times as great (about six times greater than at 84% relative humidity), the mold population about three times as great, nearly half the seeds were dead, and more than half had been invaded by storage molds.

Over the 10-day period, the daily carbon dioxide values were low, constant, and nearly equal for the samples containing 9.6 and 11.6%

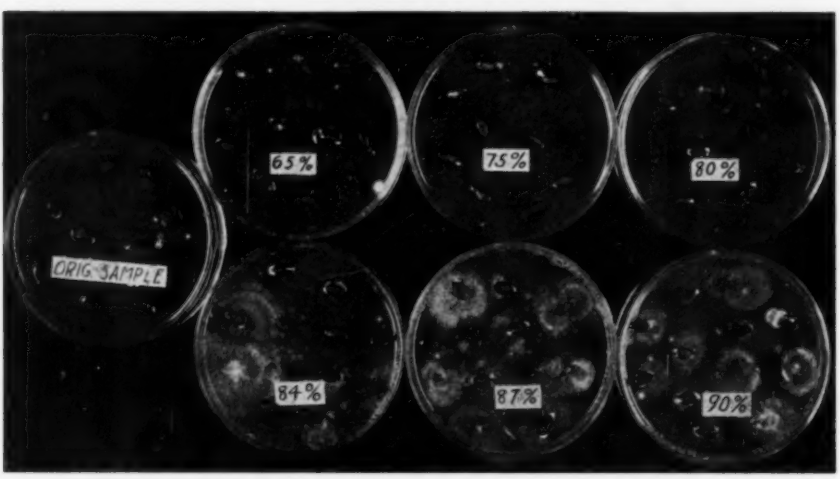

Fig. 1. Cottonseed stored at the indicated relative humidities for 10 days, then delinted, surface-disinfected and placed on malt-salt agar. Storage molds increased rapidly above 84% relative humidity.

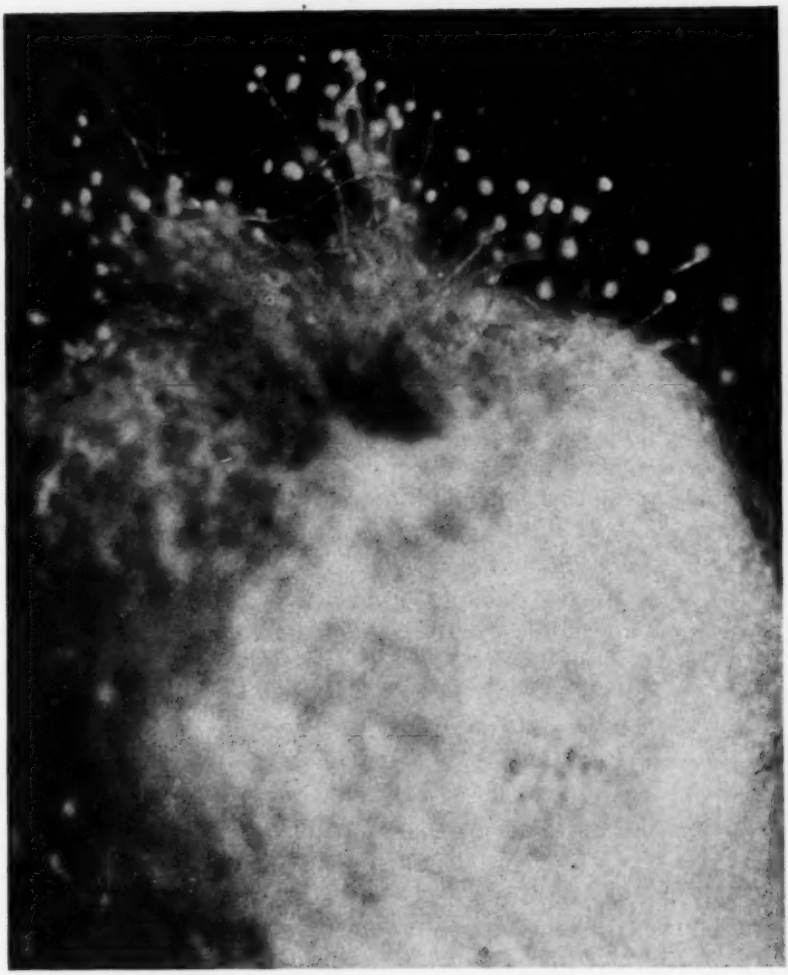

Fig. 2. *Aspergillus ochraceus* growing from basal end of cottonseed stored 10 days at 90% relative humidity. The original seed had less than 1,000 molds per gram, and none of the seed was internally infected with storage molds. After 10 days at 90% relative humidity, the seed had about 350,000 molds per gram and typical storage molds were present internally in more than 50% of the individual seeds.

moisture. The daily values for the samples at 13.4 and 15.2% moisture were somewhat higher and increased slightly with time. The samples maintained at 17.4% and 19.7% moisture had somewhat higher initial respiration rates than those at lower moistures and the daily rates markedly increased as the trial progressed to give carbon dioxide–production rate curves similar in shape to a microbiological growth curve.

The samples which respired at 9.6, 11.6, 13.4, and 15.2% moisture exhibited respiratory quotients of 0.6 to 0.7, whereas the daily respiratory quotients of the samples maintained at 17.4 and 19.7% moisture

increased with time and approached 1.0 at the end of the trial. This tendency of the respiratory quotients to increase when conditions are favorable for mold growth has been observed in similar experiments with soybeans and wheat (16, 19). The increasing respiration, increasing fat acidity, and decreasing viability associated with mold increase were essentially the same as have been found in similar tests (16, 18, 19) with other types of seeds stored under similar conditions. The species of molds that invaded the seeds were also essentially the same as in previous studies with other grains.

Mold sporophores were easily visible to the naked eye on seeds that had been stored at 90% relative humidity for only 10 days, as shown in Fig. 2. As will be described later, the structure of cottonseed enables molds to gain easy access to the interior of the seed, and the principal mold development is often *within* the seed coat, not outside, so that mere visual examination of the seed is an unreliable criterion of moldiness. However, in the test just described, mold sporophores were numerous and obvious to the naked eye on some of the seeds stored at 90% relative humidity.

Effect of Differential Aeration on Respiration, Fat Acidity, and Mold Population. Nine hundred grams of nondelinted seed were conditioned to 19% moisture and divided into six subsamples. Five of these were aerated at 30°C. for 13 days with 250 to 2,000 ml. of air per 24 hours, while the sixth subsample received 250 ml. of nitrogen (tank nitrogen containing approximately 0.3% oxygen). The relative humidity of the gases passed through the seed was maintained at 90%; at the end of 13 days' storage, the moisture content of the six subsamples varied from 19.1 to 19.6%. Carbon dioxide production, free fatty acids, total mold population, percentage of seeds with internal molds, and seed viability at the end of the 13-day storage period are presented in Table II and typical internal molds are illustrated in Fig. 3.

The daily respiratory rates for the sample which received nitrogen (containing about 0.3% oxygen) were practically constant throughout the 13-day trial. As the effluent nitrogen contained from 4.4 to 4.8% carbon dioxide, some anaerobic respiration occurred. The samples which received 250 and 500 ml. of air per day showed increased respiratory rates until the sixth and seventh days after which the respective rates were nearly constant. Between the sixth and thirteenth days the effluent air from the sample aerated at 250 ml. per day contained approximately 19.0% of carbon dioxide and that from the sample aerated at 500 ml. contained from 14.5 to 16.7% of carbon dioxide. The samples aerated with 1,000, 1,500, and 2,000 ml. of air per day gave increasing respiratory rates throughout the entire 13-day trial, although the rates for the sample which received 1,000 ml. of air per

day showed a tendency to level off during the last day of the trial. At this time, the effluent air contained 13.4% of carbon dioxide. The respiratory rates for the three samples receiving the highest aerations accelerated in a very similar manner until the sixth day when rate of acceleration for the sample receiving 1,000 ml. per day began to lag behind the other two. The effluent air from this sample contained 8.5% of carbon dioxide on the sixth day and 13.4% on the thirteenth day, and these carbon dioxide levels therefore partially inhibit respiration. The carbon dioxide rate curves for the samples aerated with 1,500 and 2,000 ml. per day were parallel and accelerated through the entire trial, although the rate of acceleration somewhat decreased

TABLE II

EFFECT OF DIFFERENTIAL AERATION ON RESPIRATION, FAT ACIDITY, AND MOLD POPULATION IN COTTONSEED STORED AT 19% MOISTURE AND 30°C., FOR 13 DAYS

Number	Aeration		Respiration[1]	Fat acidity		Germination	Molds		Kinds of molds
				As oleic	Acid value[2]		Total number[3]	Internal infection[4]	
	Gas	*ml.*	*mg.*	%	*mg.*	%		%	
Original seed	—	—	—	0.4	7.8	97	1,000	6	
1	Air	2000	2186.9	12.2	242.2	41	1,023,000	100	*Aspergillus glaucus* chiefly
2	Air	1500	2246.5	11.0	219.1	40	660,000	92	*Aspergillus glaucus* chiefly
3	Air	1000	1858.2	9.1	181.0	47	230,000	100	*Aspergillus glaucus* chiefly
4	Air	500	1398.1	4.8	95.2	66	240,000	100	*Aspergillus glaucus* chiefly
5	Air	250	838.3	2.7	53.0	81	—	98	*Aspergillus glaucus* chiefly
6	Nitrogen	250	198.8	0.5	9.1	44	<1,000	46	Probably *Glomerella gossypii*

[1] Total mg. CO_2/100 g. dry matter in 13 days.
[2] Expressed as mg. KOH/10 g. of oil.
[3] Molds per gram.
[4] Percentage of seeds with internal molds.

after the eleventh day. Under the conditions of this experiment 1,500 ml. of air per day were adequate to obtain maximum respiration of 150 g. of cottonseed. The results indicate that carbon dioxide concentrations above about 8.5% exert an inhibiting effect upon carbon dioxide production.

The mold population decreased somewhat with decreasing aeration, although even 500 ml. of air per 24 hours were sufficient to permit considerable increase in both molds and free fatty acids. In the sample which received nitrogen, the respiration was low, fatty acids scarcely increased, and no typical storage molds developed, but at the end of the test nearly 50% of the seeds were infected with an unidentified white mold. *Glomerella gossypii* and *Fusarium vasinfectum* were

later found to be present in many of the seeds used in these tests. These fungi are common seed-borne parasites of cotton (3), and the fungus which grew from the seeds stored under nitrogen resembled, in gross cultural characteristics on malt-salt agar, that which was later positively identified as *G. gossypii*.

In this test, some of the seed that bore more than 200,000 molds per gram could not be distinguished, by external examination with the naked eye, from the original seed that had fewer than 1,000 molds per gram. At the risk of belaboring an obvious point, it might be stated

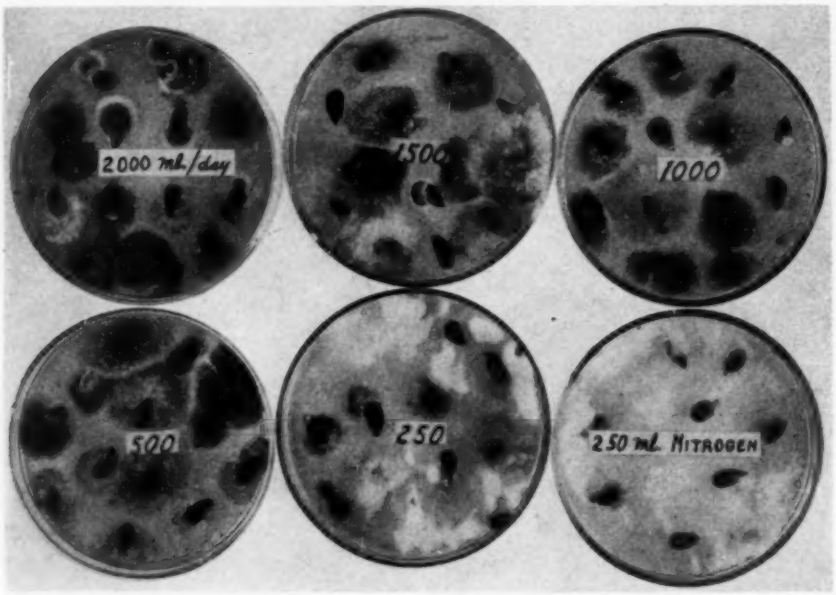

Fig. 3. Cottonseed stored at 90% relative humidity for 13 days, aspirated with the amounts of air (or nitrogen) in ml. per day indicated by the labels. After 13 days the seeds were delinted, surface-disinfected, and placed on agar. Typical storage molds were present in all seeds except those which received 250 ml. of air or nitrogen. No internal storage molds were found in the latter.

that this is additional evidence that relative moldiness of the seed cannot be accurately determined by visual examination.

Respiration, Temperature Increase, and Mold Development in Adiabatic Respirometer, with and without Thiourea as a Mold Inhibitor. Nondelinted seed was conditioned to 18% moisture, replicate lots of 100 g. each were weighed out, and to half of these 1.0 g. of finely powdered thiourea per 100 g. of dry seed was added to inhibit molds. Thiourea was chosen because, in tests of more than 100 compounds by Milner, Christensen, and Geddes (17), it effectively inhibited molds on wheat at a comparable moisture content, and did not greatly reduce viability of the seed. The samples were aerated for 23 days in the

adiabatic respirometer described by Milner and Geddes (16) at the rate of 2,000 ml. of air per 24 hours. Carbon dioxide production and temperature were measured daily and, at various intervals, samples were removed for determinations of mold population and fat acidity. The results are presented in Table III.

The nontreated seed heated more rapidly than that treated with thiourea, and, in both, the mold increase was roughly proportional to the temperature rise. The nontreated sample attained a temperature of 37°C. in 7 days, and then had a mold population of 470,000 per gram, while the seed treated with thiourea only attained a temperature of 33.1°C. in 16 days, and then bore 450,000 molds per gram. After 9

TABLE III

CARBON DIOXIDE PRODUCTION, TEMPERATURE INCREASE, AND MOLD POPULATION OF COTTONSEED STORED IN ADIABATIC RESPIROMETER WITH AND WITHOUT THIOUREA AS A MOLD INHIBITOR [1]

Treatment	Days stored	Respiration[2]	Temperature	Fat acidity		Molds per g.
				As oleic	Acid value[3]	
		mg.	°C.	%	*mg.*	
Untreated	7	163.9	37.0	1.2	23.9	470,000
	9	335.7	45.9	2.1	42.5	287,000
	16	77.1	65.7	2.6	52.7	0
	20	78.9	68.8	—	—	—
	23	99.3	74.8	2.9	57.0	—
Thiourea	7	21.4	26.2	—	—	—
	9	24.3	27.0	—	—	—
	16	68.9	33.1	0.4	8.5	450,000
	20	219.0	46.5	0.8	16.3	300,000
	23	88.9	57.0	1.3	26.8	0

[1] The original seed had 100 molds per gram and a free fatty acid content of 0.4% expressed as oleic acid equivalent to an acid value of 7.8.
[2] Total mg. CO_2/100 g. dry matter.
[3] Expressed as mg. KOH/10 g. of oil.

days' storage, the nontreated seed had a temperature of 45.9°C. and a mold population of 287,000 per gram, while the seed treated with thiourea required 20 days to attain a temperature of 46.5°C. and a mold population of 300,000 per gram. The chief mold present in both samples was *Aspergillus glaucus*. Previous work in these laboratories has shown that this mold gradually is eliminated after the temperature rises above approximately 40°C., and so the reduction in mold population as the temperature exceeded 45°C. was not unexpected. No molds were found on the seed after the temperature exceeded 55°C., as was to be expected, since none of the molds encountered on these seeds will survive above that temperature.

The preliminary stages of heating of the moist seed were accompanied by vigorous mold growth of the same species as has been found to accompany preliminary heating in other grains (16, 22, 23). Once the temperature exceeded 45°C. the heating process was faster than has been found in soybeans or wheat; and molds such as *Aspergillus flavus*, *A. candidus*, *A. fumigatus*, and *Mucor* sp., which grow vigorously on the latter seeds between 45° and 50°C., were not encountered on cottonseed in the present test.

That powdered thiourea, in the concentration used, failed to prevent mold growth and heating was easily explained once the seeds were sectioned and examined internally. There was little or no external mold on the seed treated with thiourea, but, after two weeks' storage,

TABLE IV

INFLUENCE OF FIVE FUNGICIDES, APPLIED AS DUSTS IN A CONCENTRATION OF 1:100, ON THE INCREASE OF MOLDS IN COTTONSEED STORED AT 19.0% MOISTURE [1]

Fungicide	Seeds with internal molds after 30 days	Internal molds per gram		Principal kinds of molds
		Storage time		
		30 days	60 days	
	%			
Control	26	11,000	800,000	Mixed flora
Arasan	92	210,000	13,000,000	Penicillium
Thiourea	70	1,500,000	14,000,000	Penicillium
Spergon	38	3,200,000	22,000,000	Penicillium
Chloramine-B	8	—	36,000	*Aspergillus candidus*
8-hydroxyquinoline	0	—	100,000[2]	*A. glaucus*

[1] The original seed had less than 1000 molds per gram and 18% were infected with internal molds.
[2] All of these colonies arose from particles of the seed coat, indicating that the seed coat itself had been invaded by mold mycelium. The colonies grew slowly, and apparently were inhibited by the fungicide remaining in the seed coat, or by that which had diffused from the seed coat into the agar medium.

many of the seeds had a heavy internal mold flora which could be seen readily with a binocular microscope in sectioned seeds. This suggests that external application of a compound known to be fairly toxic to most storage molds will not necessarily protect the seed from mold invasion nor reduce mold growth within the seed. This was proven more conclusively in the following test.

Influence of Various Solid Fungicides on the Development of Molds within Moist Seeds. Duplicate portions of nondelinted cottonseed, variety Bobshaw, obtained from Virginia, were conditioned to 19% moisture, dusted with five different fungicides, each in a concentration of one part fungicide per 100 parts of moist seed, and stored in rubber-stoppered 250 ml. Erlenmeyer flasks at room temperature for 30 to 60 days. In all cases, the amount of fungicide was in excess of that

which would adhere to the seed after vigorous shaking. The fungicides used were Arasan, Chloramine-B, Spergon, thiourea, and 8-hydroxyquinoline. Of these, thiourea, 8-hydroxyquinoline, and Chloramine-B were among the most effective mold inhibitors of more than 100 compounds tested by Milner, Christensen, and Geddes (17) on wheat seed. Arasan and Spergon are widely used and effective seed-treatment fungicides. They were included not because they were considered to be effective mold inhibitors on stored seed, but only to determine the influence of such compounds of known fungicidal potency upon storage molds.

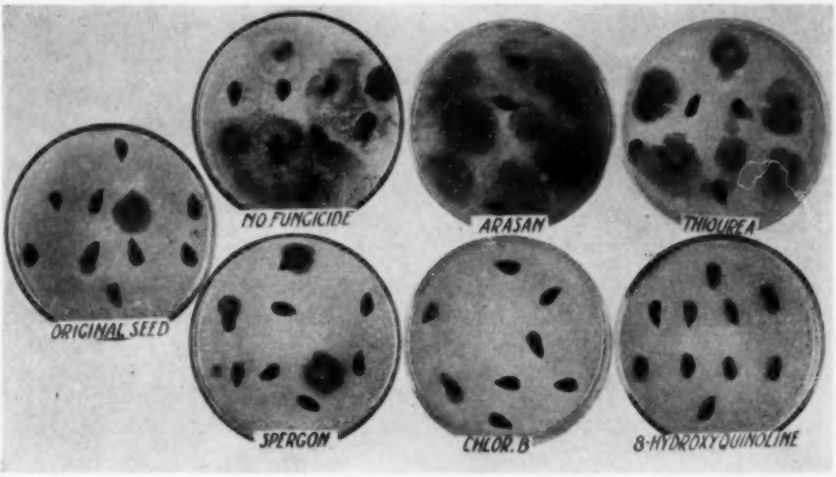

Fig. 4. Cottonseed treated with various dust fungicides (1:100) and stored 28 days at room temperature, 19% moisture, then delinted, surface disinfected, and cultured. Note that the kinds of molds infecting seed treated with thiourea differ from those from the seed treated with Arasan.

The total mold population after 30 and 60 days' storage and the percentage of seed with internal molds after 30 days' storage are shown in Table IV. None of these compounds entirely prevented mold increase, and some apparently stimulated mold growth. Some molds, especially *Aspergillus glaucus*, developed so vigorously on the seed treated with Arasan that the sporophores grew out in long tufts with visible particles of the fungicide on them. The mold population given in Table IV includes only the molds that developed internally. The seed was delinted in concentrated sulfuric acid before grinding, and this eliminated the heavy crop of external spores on some of the seeds. This treatment was necessary because, while a given compound might not inhibit molds on the seed at 19% moisture content, it might suppress their growth in agar; i.e., the potency of a given compound as determined by tests on agar plates may bear little relationship to its

potency under other conditions, a fact so well recognized as to need no emphasis. The internal mold infection in some of the seeds in this test is shown in Fig. 4.

Approximately 20 seeds treated with each compound were sectioned after 60 days' storage and examined with a binocular microscope for internal evidence of molds. Some internal mold was found in all of the

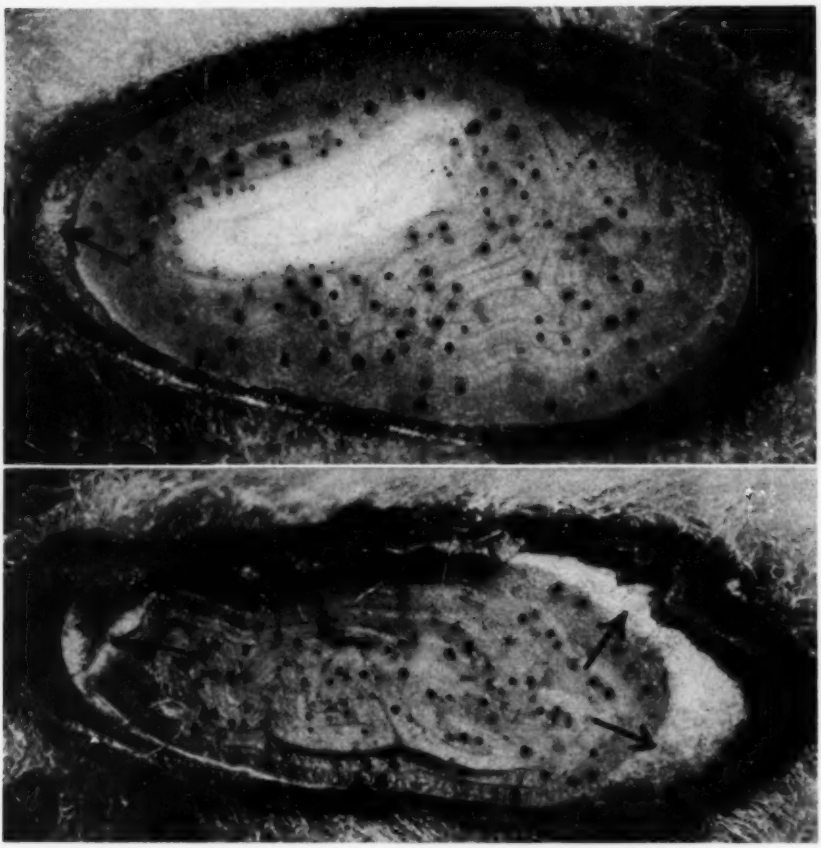

Fig. 5. Sections through seed treated with fungicide (top, Arasan; bottom, thiourea) and stored 60 days at 19% moisture. The arrows point to heavy mold development on the inner wall of the seed coat and on the embryo. The dark circular spots in the embryo are oil glands.

seeds and, in some, treated with supposedly effective moldicides, the entire inner wall of the seed coat was covered with mold sporophores. Photomicrographs of sections of these seeds are shown in Fig. 5.

That the seeds treated with 8-hydroxyquinoline, when cultured, appeared to be free of internal molds may have been due to small amounts of the compound that remained in the seed coat, sufficient to prevent the molds internally present from growing out in agar. As

evidence of this, such seed, when ground and cultured, gave rise to numerous mold colonies. All of these colonies arose from small portions of the seed coat, as determined by microscopic examination of the culture plates. These colonies appeared only after 10 to 14 days, grew very slowly, and were obviously suppressed. This suggests that the seeds treated with this compound were, at the end of the test, somewhat more moldy than the results indicated, and also that it may sometimes be rather difficult to determine whether molds are growing in seed treated with a fungicide.

It is evident from this test that compounds of even rather high toxicity to molds may be relatively ineffective in protecting moist cottonseed from invasion by storage molds. This is important because in some tests with stored cottonseed it has been assumed that treatment with a compound known to be toxic to certain fungi under certain conditions was also equally toxic to the storage molds, and that application of such compounds to the seed automatically eliminated molds. The present tests indicate that the effectiveness of a compound in eliminating molds from moist stored cottonseed can be determined only by testing the compound under the storage conditions involved, and by determining the mold population at the beginning and end of the test. Obviously, molds cannot be excluded as a factor in such studies and some sort of mold assay must be an integral part of experiments that aim to determine whether molds are involved in the deterioration of stored seeds.

Structure of Cottonseed in Relation to Mold Invasion. That the heavy, thick coat of cottonseed does not protect the interior of the seed from invasion by molds has already been suggested. The fact that some fungus diseases of the cotton plant are seed-borne, and the causal organisms have been found within the seed (3), would lead one to suspect that this seed is not so impervious to mold invasion as it seems.

There are numerous studies of embryo development and seed structure of the cotton plant, but none of them, so far as the authors have been able to determine, discuss the morphology of the seed from the standpoint of its possible invasion by fungi. In a study on this aspect of the problem mature seeds of several varieties of cotton were examined, and some plants of the Stoneville variety were grown from seed to maturity in the greenhouse to obtain seed in various stages of development.

The embryo of cottonseed develops anatropously, as has been shown by Gore (9) and others; that is, the seed is curved back against, and fuses with, the stalk on which it is borne, and through which its nourishment is obtained. As determined by dissection of numerous seeds at various stages of growth, the vein through which the seed is

nourished passes up one side of the seed and divides near the blunt end. Beneath this division of the vein there is a pore-like opening in the heavy seed coat through which a branch of the vein apparently passes. A diagrammatic sketch of a section through the basal portion of an almost mature seed is shown in Fig. 6. This is typical of all the seeds of the several varieties that have been examined. The significant fact from the standpoint of fungus invasion is that this pore in the mature seed is filled only with thin-walled parenchyma cells and presents to molds an easy access to the interior of the seed. The thin-walled parenchyma cells form a plug in the opening through the seed coat. In the immature seed, the cells of this plug are contiguous to, and continuous with, the cells of the thick endosperm that surround the embryo. As the seed matures and the endosperm is consumed, a cap of this

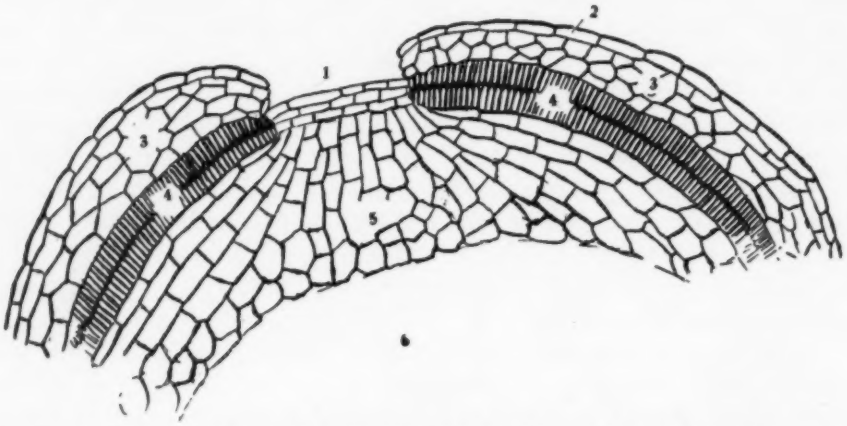

Fig. 6. Diagrammatic sketch of a section through the basal portion of a nearly mature cottonseed. Magnification about × 20. (1) represents the hilum plugged with thin-walled cells, (2) epidermis, (3) mesophyll, (4) palisade cells, (5) endosperm (which remains as a cap over the embryo), and (6) embryo.

parenchyma tissue is left at the base of the embryo. When the ripe seed dries out, the cells of this cap separate from the inner wall of the seed coat, and frequently the plug of tissue retracts from the pore in the wall. In seeds that have been delinted with sulfuric acid, the open pore in the wall often is visible to the naked eye. Somewhat magnified, it appears as shown in Fig. 7. Sometimes this pore is concealed by a few very thin layers of cells that originally made up the outer epidermis of the seed. In none of the hundreds of individual mature seeds of several different varieties of cotton that have been dissected was there anything more than a superficial covering over this pore. Thus the interior of the seed is exposed to easy infection by such molds as may have opportunity to invade it.

As evidence that molds normally enter the seed through this readily available opening, spores of *Glomerella gossypii* have been

rather commonly found in the embryo tissue beneath the pore of high-quality seeds furnished the authors by the Southern Regional Research Laboratory. In cottonseed stored at 90% relative humidity for as short a time as 10 days, molds were found sporulating vigorously *within* the seed coat adjacent to this pore. With longer storage, at

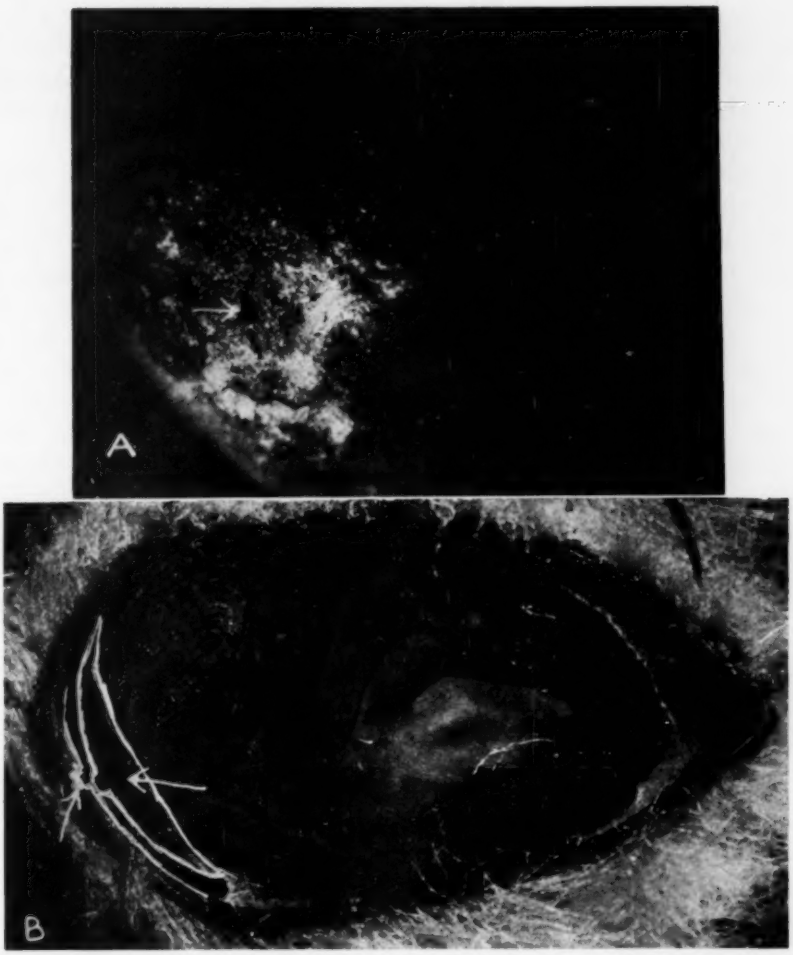

Fig. 7. A. Exterior of basal end of delinted cottonseed, showing the aperture (indicated by the arrow) in the seed coat. B. Section through a cottonseed showing the "cap" of tissue (outlined in white) which was pulled away from the seed coat, leaving an opening through the seed coat, indicated by the upper arrow.

moisture contents favorable for their development, the molds may spread over the entire inner wall of the seed coat and sporulate profusely within the seed coat.

The general impermeability of the seed coat may even promote mold development at times. Such a seed coat, of course, excludes

nonvolatile fungicides and doubtless limits the penetration of some liquid fungicides. Such fungicides may have little or no effect on molds growing within the seed coat. This helps to explain why compounds known to be toxic to molds, such as thiourea, Spergon, or Arasan, when applied to the outside of the seed, may not eliminate or even reduce mold growth within the seed. Thus, the chief difference between cottonseed and such seeds as wheat or soybeans, so far as mold invasion is concerned, is that heavily molded seeds of wheat or soybeans are likely to be outwardly and visibly moldy (although this is not always true), while cottonseed may be heavily molded internally even when it appears outwardly to be free of molds.

Respiration of Intact Seeds, Separated Meats, and Hulls. The hulls were removed by hand from approximately 400 g. of delinted cottonseed. That this resulted in relatively little injury to the embryos is shown by a germination of 85% of the separated meats, as compared with an initial germination of 95% of the intact seed. Meats and hulls were each divided into duplicate lots, and one of each was moistened with water, the other with a solution of thiourea to give one part thiourea per 100 parts of dry hull or meat. Duplicate portions of intact seed were similarly treated. The materials were stored at 30°C. for 10 days, and were continuously aerated with atmospheres of 90% relative humidity. The carbon dioxide production was measured daily, and the fat acidity, viability, and mold population of the seed after 10 days' storage. The results are given in Table V.

In this experiment, the solution of thiourea inhibited respiration and mold increase more effectively than did thiourea when applied as a dust (Table IV), but it did not eliminate molds. Respiration and mold population were considerably higher for the nontreated material than for that treated with thiourea. Molds increased slightly on the treated intact seed and on meats, but increased considerably on the treated hulls. That is, even applied as a solution, thiourea greatly reduced but did not completely inhibit mold increase. The major part of the respiration of the meats treated with thiourea must be attributed to the embryo itself, while the respiration of the hulls, both treated and untreated, must be ascribed mainly or solely to molds. The high respiration and high mold population of the nontreated meats indicate that the embryo, as well as the hull, is a favorable medium for mold increase. The test does not disclose whether the decrease in viability of the meats treated with thiourea was due to the toxicity of thiourea, to chemical processes within the embryo itself, or to molds that were invading the interior of the meats but which did not sporulate on the exterior and were not revealed in the mold assays. No attempt was made to determine the acidity of the hulls, since Karon (10) has

shown that they are essentially free of fats. Both the intact seeds and the separated meats show a close relationship between mold increase and fat acidity increase, indicating that molds may be significantly concerned in the deterioration of moist stored cottonseed.

TABLE V

CARBON DIOXIDE PRODUCTION, FAT ACIDITY, AND MOLD POPULATION OF INTACT MOIST COTTONSEED, COTTONSEED MEATS, AND HULLS, STORED AT 90% RELATIVE HUMIDITY WITH AND WITHOUT THIOUREA

(Samples stored 10 days at 30°C.)

Material	Treatment	Moisture content		Respiration[2]	Fat acidity		Germination	Molds per g.	Principal kinds of molds
		Original	Final[1]		As oleic	Acid value[3]			
				mg.	%	*mg.*	%		
Original seed	Nil	—	—	—	0.5	10.0	90	1,000	*Glomerella gossypii*
Original meats	Nil	—	—	—	0.4	8.2	85	200	*Aspergillus glaucus* (probably from contamination during sampling)
Intact seed	Nil	19.2	21.0	1116.6	4.4	92.5	64	5,500,000	*A. glaucus* and *Penicillium* sp.
	Thiourea	17.9	18.1	246.3	0.6	13.8	95	5,000	*A. glaucus* and *Penicillium* sp.
Meats alone	Nil	17.9	19.9	5443.6	14.2	301.8	0	100,000,000	*Penicillium* sp., *A. glaucus*, and *A. candidus*
	Thiourea	17.3	17.7	1057.4	0.3	5.6	50[4]	1,000	*Penicillium* sp.
Hulls alone	Nil	19.8	19.5	858.7	—	—	—	2,500,000	*Penicillum* sp. and *A. glaucus*
	Thiourea	19.5	18.9	192.4	—	—	—	140,000	*Penicillium* sp. and *A. versicolor*

[1] According to Karon (10), at 90% relative humidity the meats could be expected to have a moisture content of 18.0%, the hulls 20.0%, and the intact seeds 19.2%. The moisture content of the non-treated seeds, meats, and hulls increased during the test, probably chiefly as a result of mold growth.
[2] Total mg. CO_2/100 g. dry matter.
[3] Expressed as mg. KOH/10 g. of oil.
[4] No normal sprouts developed from these meats.

Discussion

The results clearly show that an increase in mold population in moist stored cottonseed is accompanied by increases in the production of carbon dioxide, fatty acids, and heat, as has been found to be the case by workers in several laboratories with other kinds of seeds. In dry grain, the respiratory rate is very low. As its moisture content is raised, the respiration increases very gradually until a certain critical

moisture range is reached, above which marked acceleration in the rate occurs and heating tendencies appear along with an increase in fat acidity. This sharp break in the moisture-respiration curves and concomitant increases in heat production and fat acidity, observed when continuous optimum aeration is employed, occurs at moisture values for the various grains which are in equilibrium with a relative humidity of about 75%, the lowest value at which the most xerophytic of the molds commonly found on, and in, grain will grow readily. Bacteria are not normally a factor in the spoilage of stored grain since the moisture content must approach equilibrium with an atmosphere exceeding 95% relative humidity before they will grow readily, and the authors attribute the sharp increase in respiration, heat production, and fat acidity which occurs above the critical moisture value to mold growth. That is, at moistures below the critical value, the low and rather uniform rate of carbon dioxide production is due chiefly to the physiological activity of the viable seeds, but at higher moistures, the seed respiration is supplemented by the much greater respiration of the growing molds. That the increase in fat acidity at high storage moistures is due mainly to the action of mold lipases rather than to the activation of the lipases of the cottonseed itself, as postulated by research workers at the Southern Regional Research Laboratory (1, 11, 12, 14), is indicated by the effect of differential aeration at constant moisture. Under nitrogen practically no mold growth occurred, and there was a negligible increase in fat acidity over the period of the trials. As the aeration was increased, there was a concomitant increase in the mold population and in fat acidity. That molds may produce lipase in quantity has been shown by Eyre (7), Kirsh (13), Thibodeau and Macy (24), Nagel and Semeniuk (21), and others. The researches of Nagel and Semeniuk are of particular interest in relation to the origin of the increase in fat acidity upon storing high-moisture grain. These workers grew pure cultures of nine fungi (which had been isolated from naturally molded corn) on steam-sterilized corn containing about 32% moisture and found that all the fungi increased the fat acidity of the corn. The greatest increases were produced by *Penicillium chrysogenum I*, *P. chrysogenum II*, *Aspergillus niger*, *A. flavus*, *Mucor racemosus*, and *A. amstelodami*. Unpublished work by J. J. Goodman at the Minnesota Experiment Station has also shown that many of the storage molds encountered on agricultural seeds produce lipase.

In several of the present studies, molds increased from an original population of less than 1,000 per gram to a final population of millions per gram during a storage period of only 10 to 20 days. Since several of these molds are known to produce lipase, and since cottonseed itself

has not been proven to produce lipase under the storage conditions maintained in these tests, it seems reasonable to attribute a considerable proportion of the fat acidity increase in these stored seeds to the action of mold lipase. The parts played by seed enzymes and mold enzymes in the deteriorative process admittedly can be determined only by tests in which the seeds are stored free of molds. Such tests are difficult to make, primarily because it is difficult to keep seed free of storage molds under conditions that favor their growth. To eliminate molds it is not sufficient merely to treat seed with a supposed fungicide. Regardless of the means used to eliminate or inhibit molds, one must depend upon some method of measuring mold population to determine experimentally whether molds are increasing or decreasing. The techniques used for this purpose in the experiments described here are by no means perfect, but at least they permit a fair quantitative comparison of moldiness. Neither Malowan (15), who stated that molds were not involved in the deterioration of cottonseed, in his tests, nor Altschul and his associates (1, 5), who expressed doubt regarding their role in the deterioration of this seed on storage, presented any experimental evidence to indicate whether the mold populations in the seeds they studied were increasing or decreasing. The data presented here leave little room for doubt that molds were a significant factor in the production of carbon dioxide, heat, and fatty acids in these experiments, and imply that they may have had an equally important, if unobserved, part in somewhat similar tests made by other workers.

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DETERMINATION OF THE PENTOSANS OF WHEAT AND FLOUR AND THEIR RELATION TO MINERAL MATTER [1]

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ABSTRACT

A study was made of the correlation between pentosan and ash content and the possible use of pentosan values as a criterion of flour grade. Consideration was given to methods of pentosan analysis.

The Hughes-Acree method for determining pentosans was adaptable to wheat and flour. Significant correlations between ash and pentosan content of two series of flours experimentally milled to varying degrees of purity were $r = +0.85$ and $r = +0.56$. However, clear grade flours showed lower correlations. The analyses of flour mill streams showed that low pentosan was generally associated with low ash content. Pentosan content was found generally to decrease with flour particle size. Among the individual bran layers the pentosan content was highest in the epidermis portion, and a significant correlation between ash and pentosan of $r = +0.63$ was found for epidermal tissues obtained from various wheats. No significant correlation was found for the cross layer, testa, or hyaline-aleurone layers, since the ash of these layers varied greatly among the varieties of wheat studied, whereas the pentosan content was fairly uniform.

The purposes of this investigation were to find a method suitable for routine determinations of pentosans of wheat and flour and to investigate the possibility that pentosan content is correlated with the mineral matter or ash content. Additional information concerning the distribution of pentosans in wheat and flour may lead to the practical use of the pentosan determination as a flour-grading factor.

Jacobs and Rask (5) suggested the use of pentosan content in calculating flour yield and reported pentosan values from 2.95 to 3.20% on a 13% moisture basis. Baker, Parker, and Mize (1) claim that two types of pentosans exist in wheat flour: a water-soluble type that forms a gel when oxidized and thus may have a role in baking, and an insoluble type that is present in relation to the surface area of the small starch granules.

Generally, existing methods of determination depend upon the hydrolysis of pentosans with a solution of hydrochloric acid and distillation of the furfural produced from the pentose sugars. Empirical factors are applied to the furfural determination and the results expressed as pentosans. The accuracy is limited by the yield of furfural, loss by volatilization, and destruction by overheating. For example,

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production of furfural derivatives, particularly hydroxymethylfurfural from glucose, will cause an error of overestimation. Often errors cancel one another, but results from abridged determinations require comparison with official methods to assure accuracy.

Gravimetric methods such as the phloroglucinol and thiobarbituric acid precipitations which require overnight to complete them are not adaptable to a large number of analyses. Volumetric methods have the advantages of speed and sensitivity at low concentration of pentosans. Noll and Belz (8) describe a method involving the use of hydroxylaminehydrochloride. The reaction involves the formation of α-furanaldoxime and hydrochloric acid which can be titrated with standard alkali. The work of Hughes and Acree (2, 3, 4) on the use of controlled bromination of furfural is particularly noteworthy as the method is easily adapted to quick routine analysis. Vernon and Metzner (10) modified the procedure and reported it adaptable to rye, corn, barley malt, and distillers' dried grain.

Materials and Methods

The apparatus used for hydrolysis of pentosans and distillation of furfural consisted of a 500-ml. flask fitted with a two-hole stopper. A bent tube directed toward the inside wall of the flask was connected to a separatory funnel graduated in 30-ml. intervals so that the acid concentration could be kept constant. A connecting bulb linked the flask to a condenser. The distillate was passed through a small filter and collected in a 500-ml. glass-stoppered Erlenmeyer flask marked in 30-ml. graduations.

A weighed sample was introduced into the boiling flask with some glass beads, and 125 ml. of 12% hydrochloric acid solution (by weight). Heat sources were 300 watt Goldfisch units connected in pairs in parallel circuit, giving distillation rates of 30 ml. per 10 minutes with a minimum of local charring. Distillation was continued until 360 ml. of distillate were collected, 30-ml. portions of the hydrochloric acid solution being added to the boiling flask as equivalent amounts of distillate were collected.

Studies were made of distillates from samples of bran and commercial patent flour using the phloroglucinol method as described in *Cereal Laboratory Methods* (4th ed., 1941), the hydroxylaminehydrochloride method, and a modified bromine method. The modified bromine method is as follows:

The flasks containing the distillate were closed and cooled in an ice bath to 0°C., 25 ml. of bromide-bromate solution (3 g. of potassium bromate and 50 g. of potassium bromide per liter) were introduced by a fast-delivery pipette and the flask closed quickly. The reaction was

allowed to continue for exactly 4 minutes, 10 ml. of 10% potassium iodide solution added, the flask closed and shaken gently.

The solution was titrated rapidly with 0.1 *N* sodium thiosulfate solution to a yellow color, starch indicator added, and titration continued to a colorless end point. A blank determination was made in the same manner. The pentosan content of the sample in grams equals ml. of 0.1 *N* sodium thiosulfate (blank minus titration) × 0.0082. All determinations are averages of duplicates.

The method of Shetlar, Rankin, Lyman, and France (9) was used with slight modification to remove the individual bran layers from 10 varieties of hard red winter wheat for a study of the distribution of pentosan and mineral matter. The epidermis and cross layer fractions were virtually pure, but the testa contained some contamination from these layers. The microscopic technique of Shetlar *et al.* was applied to the testa fraction for correction of the percentage of the kernel represented by epidermis, cross layer, and testa, but was not applied to the results of chemical tests.

The hyaline-aleurone fraction contained some endosperm, and all analyses were corrected to an endosperm-free basis by starch analyses since pure hyaline-aleurone is free of starch. Starch was determined by the polarimetric method described in *Cereal Laboratory Methods* (5th ed., 1947).

The epidermis, hyaline-aleurone, and endosperm fractions were ashed as recommended in *Cereal Laboratory Methods* (5th ed., 1947), but the cross layer and testa portions required special treatment. In the latter case, ash was obtained at 600°C. after pretreatment with glycerol-alcohol mixture as described in *Cereal Laboratory Methods* (4th ed., 1941). The sodium content of the ash from cross layer, testa, and endosperm samples was determined by the spectrographic method of Morris, Pascoe, and Alexander (7) and the results, calculated as sodium carbonate, were deducted from the corresponding ash values

TABLE I

COMPARISON OF THREE METHODS OF PENTOSAN DETERMINATION ON WHEAT BRAN AND FLOUR

Sample	Pentosan content		
	Phloroglucinol	Hydroxylamine-HCl	Bromine
	%	%	%
Bran	21.7	22.6	21.6
	21.1	21.7	21.2
Flour	2.90	3.14	2.96
	2.92	3.20	2.96

to correct for the treatment with alcoholic sodium hydroxide. Because of the great bulk of the epidermis and the difficulties in determining moisture, samples of this fraction were air-dried in an air-conditioned room for 48 hours to attain moisture equilibrium. The samples were stored in sealed cans until analyzed.

Samples of the original and debranned wheat varieties were milled on the Buhler mill after tempering to 15.5% moisture and the flours analyzed for ash and pentosan. The debranned wheat had the epidermis, cross layer, and testa layers removed, leaving only the hyaline-aleurone layers attached to the starchy endosperm.

Results and Discussion

The pentosan results obtained by the hydroxylaminehydrochloride, modified bromine, and phloroglucinol methods on bran of Pawnee variety wheat and commercial hard wheat flour are shown in Table I. The hydroxylamine method gave the highest results but the poorest precision. The necessity of exactly neutralizing the acid-furfural distillate with methyl orange made this method impractical for a large number of analyses.

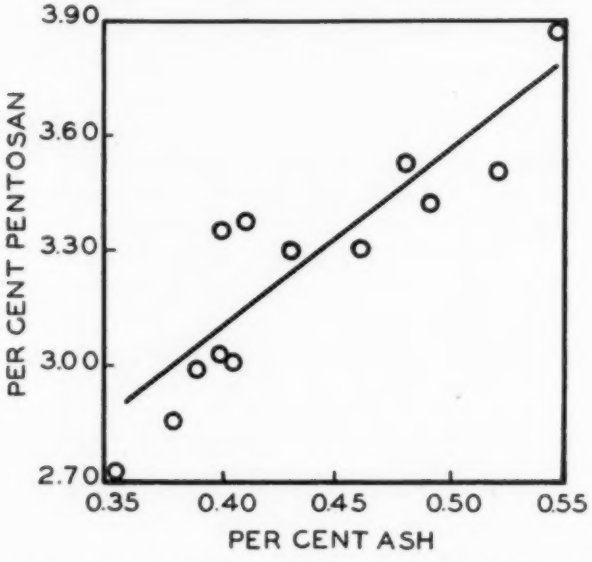

Fig. 1. Scatter diagram showing relation between pentosan and ash content of flours experimentally milled from hard red winter wheat to varying degrees of purity.

The bromine method gave comparable results to the phloroglucinol method. The difference between duplicates for the bromine method was less than 2% and the total time of determination was less than 3 hours. To obtain the maximum yield of furfural at least

360 ml. of distillate had to be collected, since marked underestimations were noted with lesser amounts. Nevertheless the bromine method was adopted for the following studies.

Pentosan determinations were made on 13 flours which were experimentally milled to varying degrees of purity from hard red winter wheat, to give samples covering a similar range in ash content to that encountered in commercial flours. Fig. 1 shows the relationship between ash and pentosan content of these flours. The significant correlation coefficient of $r = +0.85$ suggested that the pentosan value

TABLE II

ASH AND PENTOSAN CONTENTS OF FLOUR STREAMS MILLED FROM PAWNEE VARIETY HARD RED WINTER WHEAT [1]

Sample	Ash	Pentosan
	%	%
MILLED FEBRUARY 25, 1947		
Third break	0.44	2.23
Sizing	0.32	2.33
First middling	0.33	2.51
Second middling	0.28	2.41
Third middling	0.32	2.57
Fourth middling	0.36	3.00
Flour	0.40	3.00
MILLED MARCH 3, 1947		
First break	0.54	2.17
Second break	0.55	2.65
Third break	0.51	2.62
Fourth break	0.72	2.58
Sizings	0.33	2.57
First middling	0.31	2.45
Second middling	0.27	2.24
Third middling	0.30	2.38
Fourth middling	0.35	2.83
Fifth middling	0.53	3.29
First tailing	0.54	2.51
Second tailing	0.55	2.94
First and second reels	0.65	3.15

[1] Results reported on a 14% moisture basis.

might be used to predict ash content. However, 12 clear flour samples with ash contents ranging from 0.60 to 1.16% gave the nonsignificant correlation of $r = +0.14$. In flour samples having ash contents greater than approximately 0.55%, the pentosan values were found to fluctuate greatly.

Ash and pentosan analyses made on samples of certain mill streams collected on two days from the 130-sack mill at Kansas State College while operating experimentally on Pawnee variety wheat are recorded in Table II. Analogous conditions existed during both samplings.

The first break stock, which is high in ash, and the first, second, and third middling streams, which are low in ash, are among the lowest in pentosan content. Thus contradicting results appear. However, the high-ash streams such as the fourth and fifth middlings, low grade, and second tailings stock were high in pentosans. Statistical analysis of the results gave a nonsignificant correlation ($r = +0.39$) that could not be used for predicting ash content from pentosan determinations.

Ash and pentosan analyses of the flour produced from 10 varieties of hard red winter wheat showed a slightly significant correlation of $r = +0.56$ between the two determinations. However, flour from the same wheats milled after being debranned gave no significant cor-

TABLE III

ASH AND PENTOSAN CONTENT OF HYALINE–ALEURONE LAYER AND ENDOSPERM FOR SEVERAL WHEAT VARIETIES [1]

Variety	Ash			Pentosan		
	Hyaline-aleurone	Flour from debranned wheat	Flour from original wheat	Hyaline-aleurone	Flour from debranned wheat	Flour from original wheat
	%	%	%	%	%	%
Tenmarq	7.26	0.56	0.59	23.5	2.45	2.84
Wichita	8.38	0.59	0.54	23.3	2.31	2.84
Comanche	8.11	0.65	0.55	21.0	2.21	2.74
Pawnee	5.70	0.62	0.56	22.9	2.04	2.49
E. Blackhull	6.05	0.52	0.53	23.9	2.51	2.88
Kawvale	4.78	0.55	0.49	23.6	2.37	2.86
Westar	4.73	0.52	0.44	25.0	2.40	2.62
Triumph	6.01	0.51	0.45	26.1	2.14	2.69
Red Chief	6.59	0.55	0.49	25.3	2.37	2.94
Chiefkan	6.72	0.53	0.53	23.8	2.49	3.02

[1] Results reported on a 14% moisture basis.

relation between ash and pentosan content. The pentosan values were consistently lower than previously encountered in flours of similar ash range.

The milling characteristics of the debranned wheat differed from the original wheat, and the absence of the outer bran layers from the wheat has reduced the pentosan content of the flour as shown in Table III. Baker *et al.* (1) reported approximately 0.06 g. of soluble pentosans per 5 g. of flour. This is about twice the difference between the pentosan values of the flour from the original and debranned wheat. The reduction in pentosan content of the endosperm from debranned wheat probably results from the combination of conditions including less contamination by bran layers, since the epidermis, cross

layer, and testa are removed, and the loss of some soluble pentosans during the process of debranning.

Relation of Particle Size to Pentosan Content. A hard red winter wheat flour of 0.47% ash content was divided into eight particle size groups by the method of Wichser *et al.* (11) and the eight fractions were analyzed for pentosans. As shown in Fig. 2, no consistent relationship between particle size and pentosan content was found. The 105 to 88 μ fraction contained the most pentosan and, in general, lower values were found as the particle size decreased except for the 61 to 53 μ range. Microscopic examination of this fraction did not show excessive amounts of bran contamination.

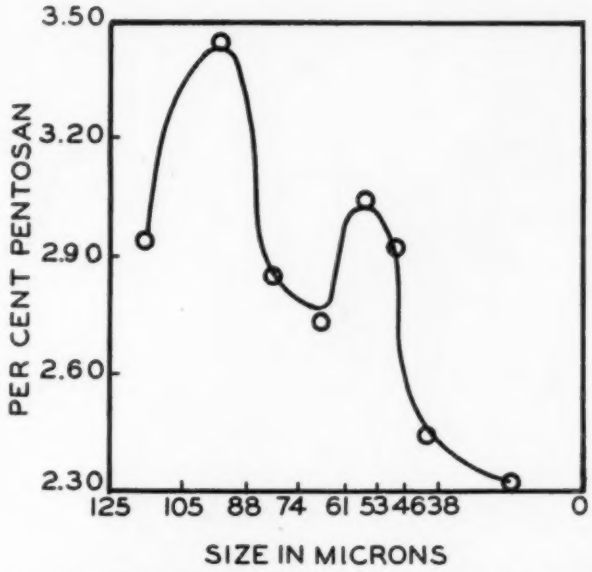

Fig. 2. Relationship between particle size and pentosan content for hard red winter wheat flour.

Baker, Parker, and Mize (1) reported 33 times as much water-insoluble pentosan in the small starch particles as in the large starch granules. MacMasters and Hilbert (6) also reported about 4% pentosans in the small granule fraction of Turkey wheat flour. In the present investigation an effort was made to study the small granular fraction of flour by employing a sifting method, but no attempt was made to separate starch granules. It was assumed that when a substance of heterogeneous particle size is sifted, the smaller particles pass through the sieve openings first. On the basis of this assumption and the results of previous investigators (1, 6) the first material through a 0–38 μ sieve should show a higher pentosan value than subsequent sievings.

To test this contention the flour used in this study was sifted 30 seconds on a Ro-Tap sifter and the throughs collected. The process was continued for another 30 seconds and thereafter repeated at one-minute intervals for a total of 5 minutes. Fig. 3 shows that the greatest amount of pentosan was found at the end of the second minute, followed by a decrease as sifting time increased. It is apparent from Figs. 2 and 3 that the smallest flour particles do not have the highest pentosan content. A satisfactory relationship between particle size and pentosan content was not established by these studies.

Individual Bran Layers and Endosperm. Pentosan and ash determinations were made on the different bran layers and endosperm

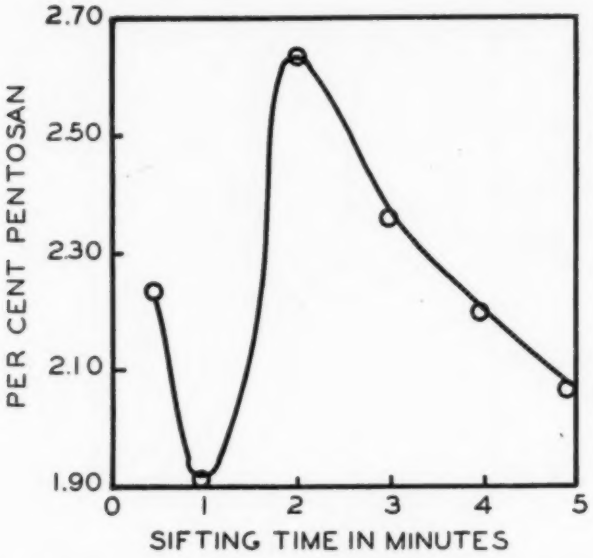

Fig. 3. Effect of sifting time on the pentosan content of flour sifted through a 400-mesh wire sieve. Particles up to 38 μ in diameter can pass this sieve.

which were separated by the procedure of Shetlar *et al.* (9). The amount of each layer, expressed both as percentages of the kernel and of total bran, is shown in Table IV. The values for each variety are the means of 10 separations and are in agreement with those of Shetlar and associates.

In separating the tissues, varietal differences were observed. For example, the epidermis of Early Blackhull required more severe treatment to remove it than was the case for the other varieties. The cross layer of Early Blackhull was easily removed, but the separation of the testa layer required extra stirring. Similar observations were made during removal of cross layer and testa from Red Chief and Chiefkan.

TABLE IV

PERCENTAGES OF INDIVIDUAL BRAN LAYERS IN 10 VARIETIES OF HARD RED WINTER WHEAT [1]

Variety	Epidermis		Cross layer		Testa		Hyaline-aleurone		Bran
	Kernel	Bran	Kernel	Bran	Kernel	Bran	Kernel	Bran	Kernel
	%	%	%	%	%	%	%	%	%
Tenmarq	3.4	23.1	0.6	3.9	0.4	2.6	10.4	70.4	14.8
Wichita	3.1	22.1	0.7	4.6	0.2	1.4	10.2	71.9	14.2
Comanche	3.5	—	0.7	—	0.3	—	—	—	—
Pawnee	3.4	23.7	0.8	5.7	0.4	3.1	9.7	67.5	14.3
Early Blackhull	3.3	22.8	1.5	10.1	0.4	3.0	9.6	64.6	14.9
Kawvale	2.7	20.8	0.8	6.3	0.5	3.7	9.1	72.4	13.2
Westar	2.6	20.0	0.6	4.8	0.3	2.5	9.6	72.7	13.1
Triumph	2.9	20.3	0.9	6.0	0.6	4.2	10.1	69.5	14.5
Red Chief	3.1	23.4	0.9	7.0	0.6	4.7	8.6	64.9	13.2
Chiefkan	3.3	—	0.5	—	0.2	—	—	—	—
Average	3.1	22.0	0.8	6.1	0.4	3.1	9.7	69.2	14.0

[1] Percentages are expressed both on basis of the total bran and of the entire kernel.

These characteristics were attributed to the waxy nature of the bran layers of these varieties. In contrast, Kawvale and Westar, because of the ease with which the layers separated, required careful handling to avoid contaminating the individual layers.

The pentosan and ash contents of 10 varieties of wheat and of their epidermal tissues are recorded in Table V. The correlation between wheat ash and pentosan content was nonsignificant ($r = +0.25$), and for the air-dried epidermis fractions a significant coefficient of $r =$

TABLE V

COMPARISON OF ASH AND PENTOSAN CONTENT OF SEVERAL VARIETIES OF WHEAT AND THE AIR-DRIED EPIDERMIS OF THE SAME WHEATS [1]

Variety	Wheat		Air-dried epidermis	
	Ash	Pentosans	Ash	Pentosans
	%	%	%	%
Tenmarq	1.75	7.4	0.90	44.0
Wichita	1.73	7.2	0.94	43.6
Comanche	1.71	7.8	0.90	43.6
Pawnee	1.60	7.1	1.00	42.7
Early Blackhull	1.83	7.4	1.20	48.4
Kawvale	1.78	7.1	1.49	46.7
Westar	1.62	6.9	1.08	46.6
Triumph	1.66	7.3	0.98	47.3
Red Chief	1.61	7.8	0.96	46.8
Chiefkan	1.70	7.6	0.93	45.0

[1] Results reported on 14% moisture basis.

+0.63 was obtained. The ash content of the epidermis varied from 0.90% to 1.49% and the pentosan values from 42.7% to 48.4%; the latter are higher than previously reported, but repeated sampling confirmed these results.

The data for the epidermal layer indicate a possible reason for the low correlation between ash and pentosan in clear flours. The range in ash content of the epidermis was only slightly higher than that of the clear flours, but the pentosan values for the epidermis are approximately 10 times as great as those for clear flour. The contamination of these flours with small amounts of bran in the form of "beeswing" or epidermis would influence the pentosan value far more than the ash content.

TABLE VI

PENTOSAN AND ASH CONTENTS OF COMPOSITE SAMPLES OF CROSS LAYER AND OF TESTA TISSUES FOR 10 WHEAT VARIETIES [1]

Variety	Ash		Pentosan	
	Cross layer	Testa	Cross layer	Testa
	%	%	%	%
Tenmarq	13.71	13.18	27.2	31.6
Wichita	11.15	13.32	30.2	29.4
Comanche	9.42	13.87	32.2	27.4
Pawnee	8.58	11.25	34.5	31.2
Early Blackhull	7.01	9.05	37.9	34.8
Kawvale	6.41	8.06	35.8	32.1
Westar	5.32	8.03	37.4	36.0
Triumph	6.38	7.40	41.5	35.8
Red Chief	6.42	6.96	36.6	34.0
Chiefkan	5.45	7.23	36.9	34.6

[1] Results reported on 14% moisture basis and the ash corrected for sodium hydroxide absorption.

The analyses of composite samples of cross layer and of testa tissues for ash and pentosans are given in Table VI. The pentosan data were consistent and in accord with previous reports. However, the ash values varied widely among varieties; Tenmarq, Wichita, Comanche, and Pawnee had notably higher ash values for these two layers. No significant correlation was found between ash and pentosan in cross layer and testa.

Table III shows the ash and pentosan results on hyaline-aleurone layers and endosperm from debranned and normally milled wheat. Sodium corrections were not necessary for the hyaline-aleurone ash values because of the acid-washing and neutralization steps, but corrections were applied to the values for flour ash. Ash and pentosan values of the hyaline-aleurone material reported in Table III were corrected for endosperm contamination. This can be done on the

basis of starch determination since pure hyaline-aleurone is void of starch. The corrected results agree with those previously reported and the values are relatively constant. The hyaline-aleurone layers constitute the major portion of the total bran and thus contribute the largest amount of ash and pentosan in the wheat kernel.

Acknowledgments

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EFFECT OF VARYING CONCENTRATIONS OF CERTAIN METALS AND THEIR SALTS ON GAS PRODUCTION AND LOAF VOLUME [1]

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ABSTRACT

Spectrographic examination of filings from the planetaries of a new 100 g. bread dough mixer indicated the presence of cadmium. Cadmium was also detected spectrographically in the ash of bread baked from dough mixed in the new mixer. These cadmium-plated planetaries materially inhibited yeast fermentation, thereby resulting in reduced gas production and loaf volume.

Gas production and bread-baking studies were carried out with varying concentrations of cadmium and its chloride along with the salts of other important metals (chromium, nickel, tin, zinc, copper, magnesium, and manganese) used in plating and in aluminum and steel alloys. Salts of lead and mercury were included for academic reasons.

The enzymes of yeast are inactivated either completely or in part by certain of these metallic salts regardless of concentrations, and by others only at specific concentrations. As little as 1 p.p.m. cadmium chloride produces a material reduction in yeast activity and loaf volume.

Chromium, nickel, or tin, and preferably the first two, are biologically safe when used for plating surfaces that come in contact with yeast-fermented doughs.

Bread from dough mixed in a new mixer recently purchased for the Hard Winter Wheat Quality Laboratory was found to be decidedly inferior with respect to loaf volume, crumb characteristics, and flavor as compared with that from an older but similarly constructed mixer. When all pin and planetary surfaces of the new mixer were covered with scotch tape, however, normal baking results were obtained. Uncovering either the bowl pins or planetary pins resulted in no damaging effects to doughs. With the planetaries bare, however, and all pins covered, subnormal loaf volumes and underdeveloped crumb grains again were obtained. Cadmium was found spectrographically in the filings from the planetaries of the new mixer but not from the old mixer. In addition, cadmium was identified (after concentration by precipitation with hydrogen sulfide) in the bread baked from doughs mixed in

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the new mixer but was not found in the bread when the old mixer was used. Simultaneously with the securing of these data, it was learned from the manufacturer that the planetaries of the new mixer had been plated with cadmium. Accordingly, bread-baking and gas production studies were carried out with varying concentrations of cadmium and its chloride along with the salts of other important metals used in plating and in aluminum and steel alloys. This paper presents the data obtained in these studies including certain comparisons of the old and new mixers.

Materials and Methods

A hard red winter flour composite of a number of typical varieties from each of two crop years was used for all baking and gas production studies. This flour, which was extracted on the Kansas State College 130 hundredweight mill, contained 11.3% protein and 0.47% ash.

Each bread dough contained 100 g. flour, 66.7 g. water, 6 g. sugar, 1.5 g. salt, 3 g. shortening, 2 g. yeast, 4 g. nonfat milk solids, 0.25 g. 120°L. malt syrup, and 1 mg. potassium bromate. All mixing except as otherwise specified was done in the old mixer—a National-Swanson-Working model. Mixing time was 2⅞ minutes at 86 r.p.m. for the old mixer and 2½ minutes at 100 r.p.m. for the new mixer. Fermentation periods and temperatures recommended by the A.A.C.C. were used. Concentrations of 0.001 mg., 0.01 mg., 0.1 mg., 1 mg., 10 mg., and 100 mg. of each of the metallic salts including cadmium chloride, chromic chloride, cupric chloride, mercuric chloride, magnesium sulfate, manganous chloride, nickel chloride, stannic chloride, zinc chloride, and lead acetate were added to the bread doughs.

Cadmium, chromium, nickel, tin, and zinc are used extensively for plating. These five metals together with copper, magnesium, and manganese are used in aluminum and steel alloys. The salts of lead and mercury were included in the experiments because of their academic interest.

The formula used in bread baking also was used in the gas production studies; 18.35 g. dough, or one-tenth of that used in bread baking, was scaled off from a regular size bread dough and placed in a pressuremeter with attached manometer. Thereafter the test was carried out as described in *Cereal Laboratory Methods*. Readings were taken every hour for 6 hours.

Results

Data in Table I show the relative toxicity of each of the various salts on loaf volume when present in the dough in concentrations of 0.001 mg. (1 part per 100,000,000) to 100 mg. (1 part per 1,000). Normal baking results were secured with concentrations of 10 mg. (1

TABLE I

LOAF VOLUMES OBTAINED WITH THE ADDITION OF VARYING AMOUNTS OF METAL SALTS TO THE DOUGH

Concentration in dough	Metal salt added									
	$CdCl_2$	$CrCl_3$	$CuCl_2$	$HgCl_2$	$MgSO_4$	$MnCl_2$	$NiCl_2$	$SnCl_4$	$ZnCl_2$	$Pb(C_2H_3O_2)_2$
mg.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
None[1]	807	807	807	807	807	807	807	807	807	807
0.001	796	—	800	804	815	805	810	825	805	799
0.01	788	818	795	799	805	800	795	830	810	808
0.1	742	819	785	806	825	810	810	810	810	808
1	580	797	798	801	800	820	796	810	761	806
10	548	805	778	674	810	808	790	794	520	789
100	543	794	460	235	805	819	745	741	761	746

[1] Loaf volume when mixed in new mixer was 674 cc. (equivalent to the effect of about 0.3 mg. or 3 p.p.m. of cadmium chloride).

part per 10,000) or less of the salts of chromium, copper (probably), magnesium, manganese, nickel, tin, and lead. Thus chromium, nickel, and tin should be satisfactory for plating surfaces that come in contact with yeast-fermented doughs. However, 10 mg. of each of the salts of cadmium, mercury, and zinc reduced loaf volumes 32%, 16%, and 36% respectively.

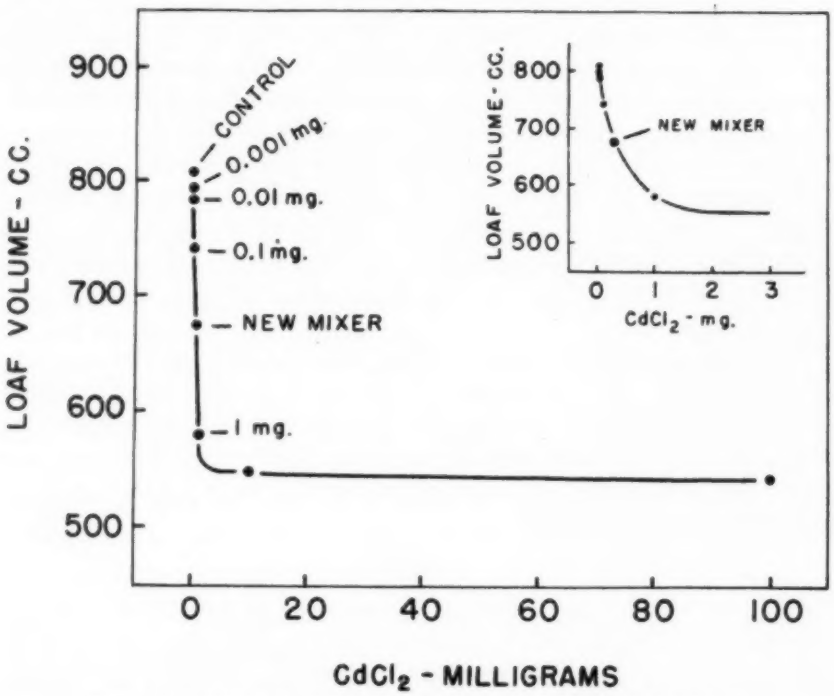

Fig. 1. Loaf volume of doughs containing varying concentrations of cadmium chloride and of dough mixed in new mixer (cadmium-plated planetaries).

At 100 mg. concentrations, mercuric chloride reduced loaf volume to 235 cc., a reduction of 71%. Enzyme activity appeared to be inhibited completely. Copper chloride, which had little or no effect on loaf volume at lower concentrations, reduced loaf volume to 460 cc., 43% below normal. Salts of nickel, tin, and lead, which at lower concentrations gave normal baking results, produced somewhat reduced loaf volumes at 100 mg. concentrations.

Several pertinent points concerning the relation of cadmium chloride concentrations in dough to loaf volume are illustrated in Fig. 1. For example, as little as 0.1 mg. of cadmium chloride (1 p.p.m.) produced a material reduction in loaf volume. When incorporated with high protein flours, even smaller concentrations of cadmium and its salts probably would result in material loaf volume reductions. Loaf volume continued to fall off rapidly, approaching the minimum as the concentration of cadmium chloride was increased to about 1.0 mg. Thereafter loaf volume decreased more slowly, the minimum volume of 550 cc. being attained with only 2 mg. cadmium chloride (Fig. 1 inset). Additional increases up to 100 mg. produced no further change in loaf volume. It should be noted from the inset of Fig. 1 that the loaf volume of 674 cc. for the new mixer corresponds to that expected for dough containing 0.3 mg. cadmium chloride or about 1.85 parts cadmium per million parts of flour.

The greatly reduced loaf volumes obtained from doughs containing cadmium, its chloride, or salts of copper, mercury, and zinc were accompanied by underdeveloped crumb grains and flavor. These results strongly indicated that dough fermentation had been seriously impaired. Accordingly, gas production studies were carried out on doughs containing 0 mg. (control), 1 mg., 10 mg., and 100 mg. of cadmium chloride and other important salts of metals used in plating and in steel and aluminum alloys.

Gas production data for the doughs containing 1 mg. of each of the various salts and for one dough mixed in the new mixer without any addition of salts are shown in Fig. 2. The only subnormal gas production curves were produced by mixing dough in the new mixer and by adding cadmium chloride to dough mixed in the old mixer. These data show that cadmium and its chloride salt are toxic to yeast fermentation. It is pertinent to note that of the five important plating materials studied (tin, nickel, chromium, zinc, and cadmium), only cadmium was toxic at 1 mg. concentration. The data are in good agreement with the loaf volume results.

Gas production data for doughs containing 10 mg. and 100 mg. of four salts, cupric chloride, mercuric chloride, zinc chloride, and cad-

mium chloride, together with those for the control and new mixer are plotted in Figs. 3 and 4, respectively.

Gassing data at the 10 mg. level (Fig. 3) are in excellent agreement with the loaf volume data in that they are about normal for cupric

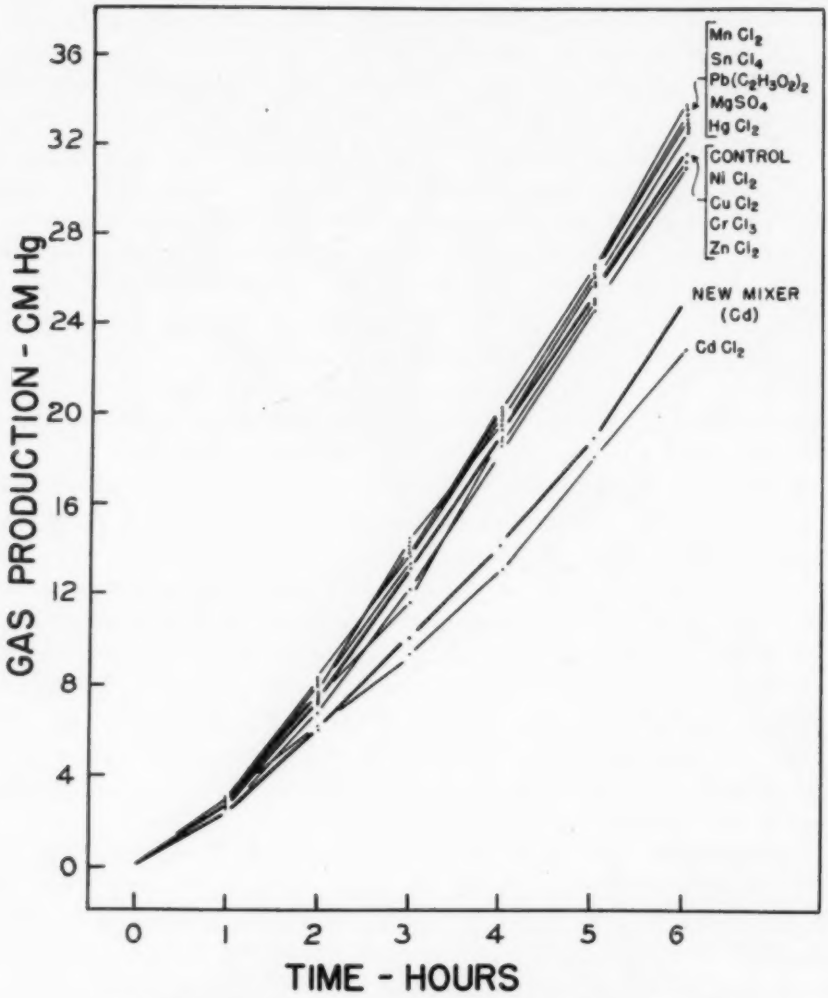

Fig. 2. Gas production of doughs containing 1 mg. concentrations of certain salts and dough mixed in new mixer (cadmium-plated planetaries).

chloride, materially below normal for mercuric chloride, and far below the control for cadmium chloride and zinc chloride.

The loaf volume and gassing data at the 100 mg. level (Table I and Fig. 4) are not only in good agreement but are of particular interest as related to the effects of lower concentrations. For example, zinc

ions affected the gas production and loaf volume in a strange manner. Concentrations of 0.001 mg. to 0.1 mg. of zinc chloride had no effect on loaf volume beyond experimental error. Addition of 1 mg. caused the loaf volume to fall slightly and gave a gassing curve (Fig. 2) that is

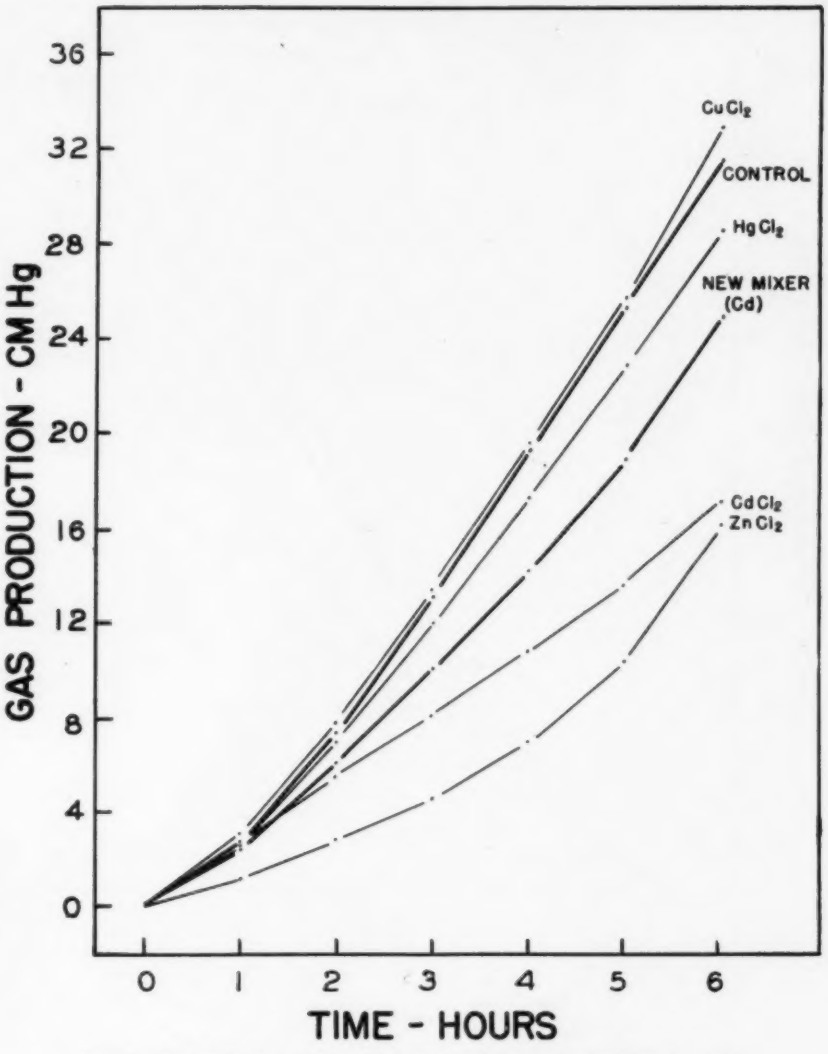

Fig. 3. Gas production of doughs containing 10 mg. concentrations of certain salts and dough mixed in new mixer (cadmium-plated planetaries).

only slightly below the control; whereas 10 mg. reduced loaf volume (Table I) to 64% normal and gas production (Fig. 3) to one-half normal. Both these values are lower than those produced by the addition of 10 mg. of cadmium chloride, the most effective inhibitor at

the lower concentrations. When 100 mg. of zinc chloride was added to the dough, however, gas production (Fig. 4) was above and loaf volume (Table I) somewhat below normal. The slightly subnormal loaf volume is assumed to be the result of excessive dough development

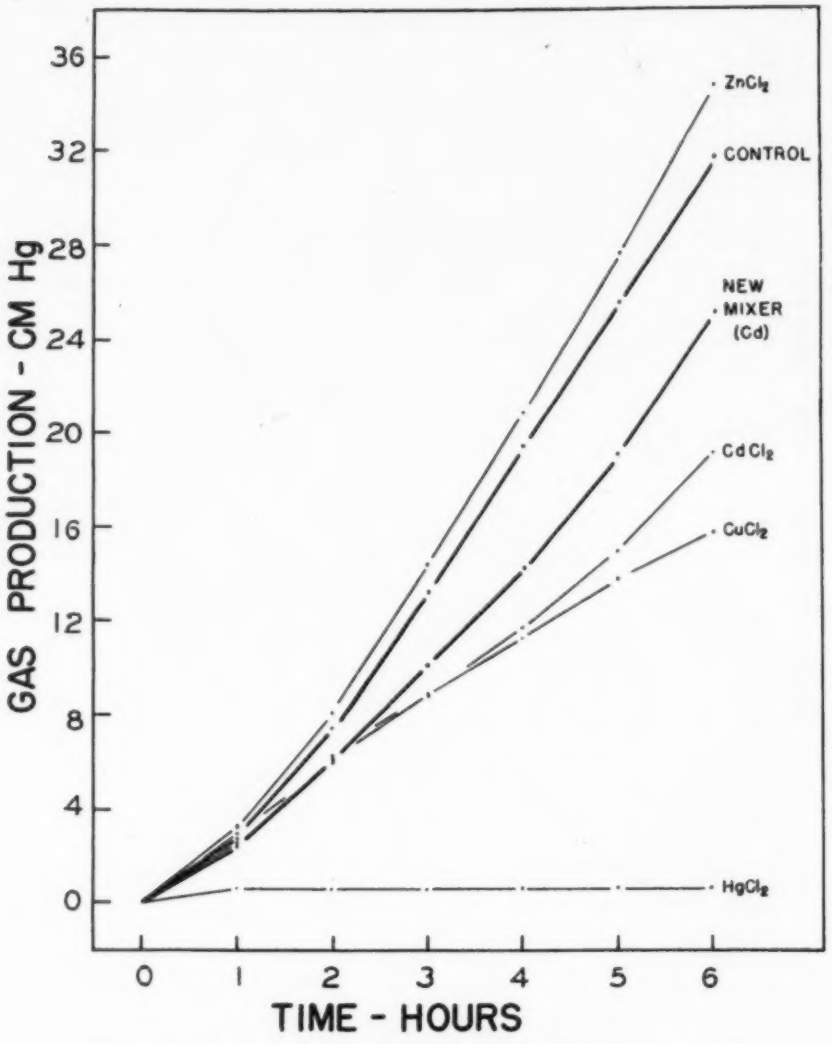

Fig. 4. Gas production of doughs containing 100 mg. concentrations of certain salts and dough mixed in new mixer (cadmium-plated planetaries).

due to above normal yeast activity. Thus at concentrations below 1 mg., the zinc ion does not act as an inhibitor; at higher concentrations it is a potent inhibitor; and at still higher concentrations yeast activity is accelerated instead of inhibited.

A large concentration of copper ions is necessary to inhibit yeast activity and reduce loaf volume. Increments of 1 mg. and 10 mg. did not affect gas production or loaf volume materially. Addition of 100 mg. cupric chloride, however, decreased gas production 50% and loaf volume 43%. These reductions are greater than those resulting from the same concentration of cadmium chloride.

Mercuric chloride, like all other salts studied excepting cadmium chloride, did not affect baking and gassing of doughs containing 1 mg. increments. A concentration of 10 mg., on the other hand, lowered gas production about 10% and loaf volume 16%; whereas 100 mg. mercuric chloride apparently stopped all enzyme activity. At the end of 72 hours the total gas produced in a dough containing 100 mg. mercuric chloride was only slightly greater than that produced after 6 hours (Fig. 4), and the dough still remained as a compact ball, indicating that proteolytic enzymes also were inactivated. The loaf volume of 235 cc. was very little larger than the volume of the dough out of the mixer. Thus zinc chloride and mercuric chloride are markedly different and each differs considerably from cadmium chloride, the only salt that inhibited yeast activity at low concentrations and whose inhibitory activity leveled off at about the same concentration where that of other salts began.

Discussion

It is well known that some enzymes involved in carbohydrate metabolism and found in wheat flour and yeast are activated whereas others are inactivated by metallic ions. For example, the activity of phosphoglucomutase is increased by Mg^{++}, Mn^{++}, or Co^{++} (6). Similarly, hexokinase, phosphatase, (enol)-phosphopyruvic acid, and carboxylase require Mg^{++} (6). β-h-Fructosidase (4) and carboxylase (5), on the other hand, are inhibited by the heavy metal ions Ag^{+}, Cu^{++}, and Hg^{++}. It is necessary to refer to enzyme systems other than those involved in yeast fermentation to acknowledge previously reported effects of Cd^{++} on enzyme activity. For example, cadmium ions inactivate the amidases histidase (3) and urease (6). Previously inactivated arginase (another amidase), on the other hand, is activated by Cd^{++} (2). The effect of Cd^{++} on the activity of certain phosphatases is interesting; glycerophosphatase is inactivated, metaphosphatase is activated, and pyro- and polyphosphatase are not affected (1). Thus, the ions of cadmium could conceivably inhibit one or more enzymes involved in yeast fermentation.

Much work to be done relates to the nature of enzyme inhibition in yeast fermentation by the salts of cadmium, zinc, copper, mercury, and others, and the specific enzymes that they inhibit. The effect of

metallic salts on *chemically leavened* baked products also should be investigated.

It is quite probable that critical parts of some commercial and household equipment have been plated with cadmium which would impair the quality of yeast-fermented products.

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EVALUATION OF DIASTATIC ACTIVITY OF MALT-CONTAINING LIQUIDS BY A MODIFIED LASCHÉ TEST [1]

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ABSTRACT

The Lasché procedure for determining amylase activity, which is based on the time required to hydrolyze a given weight of starch to the achromic point at 62.5°C., has been modified by varying the amounts of starch and diastatic liquid so that a wide range of activity can be covered. *Converting Capacities* from 0.02 to 200 can be evaluated from the equation $100\ S/Lt$ where S equals the grams of starch converted to the achromic point, L is the volume (ml.) of diastatic liquid, and t the conversion time (minutes). *Acting* or *apparent Converting Capacity* is determined when the digestion is conducted at the pH of the diastatic liquid, whereas *maximum* or *potential Converting Capacities* are obtained at the optimum pH of 4.9–5.2 (at 62.5°C.). The optimum temperature for the conversion is 62.5°C. No serious thermal inactivation occurs at this temperature if the conversion time does not exceed 8 minutes. In the presence of maltose, thermal inactivation at high temperatures is less pronounced. The modified method permits a comparison of the enzyme activities of liquids from any phase of distillery operation or with the original activity of the malt which is used.

The degradation of the starch molecule by the action of malt amylases is accomplished in three major but overlapping steps, liquefaction, dextrinization, and saccharification, all of which have been

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utilized for evaluating amylase activity by measuring respectively the change in viscosity of the substrate (starch paste), the rate of dextrinization of soluble starch, and the rate of maltose production during the initial phase of the enzyme action. Within certain limitations, all three methods are suitable for evaluating the strength of solutions containing alpha- and beta-amylase in the same proportion, but none of them will give a complete picture of the various properties of a given amylase solution.

Methods measuring the dextrinization activity offer advantages in speed and simplicity. One type developed by Sandstedt, Kneen, and Blish (5) is specific for malt alpha-amylase; the other type, the Roberts method (4) and its numerous modifications by Wohlgemuth (6), Lasché (3), and Foth (2), evaluates the over-all amylase activity. The method recommended by Lasché was specially designed for evaluating distillers' malt, and, as standardized by de Becze (1), is still widely used in the United States for this purpose. The conversion temperature (62.5°C.) used in this method approximates that prevailing during the initial stages of distillery mash conversion by malt.

In the Lasché test 5 g. of soluble starch are digested in 100 ml. of aqueous solution at 62.5°C. by an infusion of 0.55 g. of ground malt. The quality of the malt is evaluated by the number of minutes necessary to reach the achromic point. This definite stage of the starch degradation is detected by the addition of 0.001 *N* iodine solution to spot plates containing samples of the digesting solution taken at intervals of 0.25 minute.

There is a great need for a simple, rapid method for evaluating amylase solutions of widely varying activity on a single scale. The experiments reported here were undertaken to develop such a method, based on the conversion time determined by the Lasché test, and to demonstrate its accuracy and use in distillery practice.

Materials and Methods

To cover the wide range of amylase concentrations encountered in distillery processes, the Lasché test has been modified to make it a more elastic method by varying the amounts of soluble starch and of amylase solutions so that the conversion time will fall between 2 to 10 (preferably 3 to 7) minutes. The selection of appropriate values for these variables permits the measurement and expression of the enzyme activity of all malt-containing liquids in the fermentation industry. The enzyme concentration is expressed by the *Converting Capacity* (*C*) of the liquid which is equivalent to the grams of soluble starch converted to the achromic point by 100 ml. of the amylase solution under test during one minute at 62.5°C. and pH 4.9–5.2. The enzyme

activity scale ranges from 0.02 to 200 and may be divided into three parts corresponding to the activities of "strong" (malt infusions and malt extracts), "medium" (cooker mash, day-old beer, sweet yeast mash), and "weak" (two- and three-day-old beers, sour yeast mash) enzyme solutions. For a given solution C is constant and is in direct proportion to the enzyme concentration.

The procedure for determining the *Converting Capacity* (C) of a liquid begins with the selection of the amounts of enzyme solution (L) and starch (S) to be used. The conversion time (t) is then determined, and, having the values of t, L, and S, the *Converting Capacity* (C) is calculated.

Modified Lasché Procedure

REAGENTS:

Starch solution—Merck's Soluble Starch, prepared according to Lintner, is used. For accurate results a freshly prepared starch solution must be used for each series of determinations. The required quantity of starch is placed in a beaker and mixed with one-third of the total volume of distilled water which is required to make the desired quantity of solution. The remaining water is heated to boiling in another beaker, added to the starch slurry with stirring, and the resulting mixture brought to boiling. Upon cooling, the solution is made up to the required volume.

Iodine Stock Solution, 0.1 N—13.5 g. of iodine is dissolved in a solution of 24 g. of potassium iodide in 200 ml. of distilled water and diluted with distilled water to 1 liter.

Iodine Solution, 0.001 N—This reagent should be freshly prepared (the same day when used) by diluting 1 ml. of a 0.1 N stock solution to 100 ml. with distilled water.

Sodium Hydroxide Solution, 0.1 N

Sulfuric Acid Solution, 0.1 N

Acetic Acid Solution, 15%

PROCEDURE:

1. *Preparation of the materials.* If the substance to be tested is a liquid, no preparation is required; if it is a paste or viscous syrup, such as a malt extract, it must be diluted. Solid materials, such as malt, must be pulverized and extracted with water. Solutions containing undissolved solids must be allowed to settle, centrifuged, or filtered.

The amylase solutions must not, of course, be heated and preferably should be tested immediately; however, if necessary, they may be stored in a refrigerator for a few hours.

2. *Determination of conversion time.* The amount of soluble starch may be varied from 0.5 g. to 10.0 g. (preferably 1.0 to 5.0 g.) and the amount of enzyme solution from one to 50 ml.; the total volume of the digest is always brought to 100 ml. An appropriate volume of starch solution to give the estimated amount of starch required for the material under test is pipetted into a 250 ml. electrolytic beaker which is placed in a water bath at 62.5°C. If necessary, distilled water is then added so that the total volume, after addition of the amylase solution under test, will be 100 ml. When the starch solution has attained 62.5°C., pipette in the estimated amount of diastatic liquid with thorough mixing and note the time. When more than 10 ml. of the amylase solution is required, adjust its temperature to 62.5°C. immediately before its addition to the starch solution. Place a glass tube in the digest to serve as a sampler. Add two drops of 15% acetic acid solution to each recess in two adjacent columns of a spot plate. After the digestion has proceeded 3 minutes, transfer two drops to each of the two upper recesses in the spot plate; add immediately two drops 0.001 *N* iodine solution to the left recess. Test samples every 30 seconds until the achromic point is approached when the interval is reduced to 15 seconds. The color changes from blue through purple, reddish brown, and brownish yellow to the yellow color characteristic of the iodine solution. After the achromic point has been passed, add two more drops of the standard iodine solution to each recess in the left hand column and four drops to each recess in the right hand column and determine the achromic point. The time in minutes which has elapsed after the addition of the enzyme until the starch digest assumes the constant color of the following samples is the conversion time, *t*. Cool the digest to room temperature and determine its pH.

3. *Calculation of Converting Capacity.* The *Converting Capacity* (*C*) is calculated from the equation:

$$C = \frac{100\,S}{L\,t}, \qquad (1)$$

where *S* is the quantity of starch (dry basis) in grams present in the digest, *L* is the volume of amylase solution in ml., and *t* is the conversion time in minutes.

NOTES:

1. *Selection of the proper amount of starch and enzyme solution.* For "strong" diastatic liquids (*C* = 5.0 to 200), 5 g. of starch are used. The volume of diastatic liquid which should be taken for analysis can be estimated if its nature, and hence the approximate

value of C, is known. For example, if the *Converting Capacity* (C) of a malt infusion is 20 and the desired conversion time is 6 minutes, a suitable volume, as computed from equation (1), $L = 100\ S/tC = 100 \times 5/6 \times 20 = 4.2$ ml. Accordingly 4.0 ml. is taken for analysis; this volume will be satisfactory for diastatic liquids with *Converting Capacities* between 15 and 30.

For solutions with "medium" activity (C = 0.25 to 5.0), 1.0 g. of starch is employed and the volume of enzyme solution is estimated in the manner already described.

For "weak" solutions (C = 0.02 to 0.25), 50 ml. of the solution are used and the amount of starch, S, is estimated from equation (1).

2. *Adjustment of pH.* *Converting Capacities* measured at two levels of hydrogen ion concentration are of general interest, namely: (1) at the optimum pH (4.9 to 5.2 at 62.5°C.), which gives the *maximum*, or *potential, Converting Capacity;* and (2) at the pH existing in the original enzyme solution which gives the *acting* or *apparent Converting Capacity.* When the pH of the digestion mixture is not optimal, the required amount of 0.1 N sodium hydroxide solution or 0.1 N sulfuric acid solution to obtain pH 4.9–5.2 is predetermined and a digestion carried out at the optimum pH. Individual adjustments of the hydrogen ion concentration, instead of using a general buffer solution, seem more appropriate because of the great variety of diastatic preparations which are to be tested.

Results and Discussion

Influence of Concentration of Reactants on Converting Capacity. Two solutions, different in origin and character, a malt infusion and a cooker mash, were employed to study the effect on *Converting Capacity* of varying the quantities of starch and enzyme solution. Three series of experiments were carried out.

In Series A, the amount of enzyme solution (L) was varied while the amount of starch (S) was kept constant. In Series B, both L and S were varied but their ratio was kept constant. In Series C, S was varied while L was kept constant. The conversion time (t) in all experiments was determined at both the natural and the optimum pH.

The results, which are summarized in Table I, show that the equation for *Converting Capacity*, $C = 100\ S/Lt$, holds over a wide range of starch and enzyme concentrations. Thus, in Series A, the conversion time, or reaction velocity, is inversely proportional to the enzyme concentrations in accordance with the following equation derived from (1):

$$\frac{S}{t} = \frac{LC}{100} = kL. \qquad (2)$$

TABLE I

Effect of Varying the Quantity of Starch and Enzyme Solution on the Converting Capacity of Malt Infusion and Cooker Mash

	Liquid (L)	Starch (S)	Natural pH	Conversion time (t)	Added N/10 H_2SO_4	Adjusted pH	Conversion time (t)	Converting Capacity
	ml.	*g.*		*min.*	*ml.*		*min.*	C
MALT INFUSION [1]								
Series A	3	5	5.15	10.50	0.00	5.15	10.50	15.87
	3.5	5	5.15	8.50	0.00	5.15	8.50	16.81
	5	5	5.20	6.25	0.10	5.05	6.25	16.00
	6	5	5.40	5.25	0.20	5.10	5.25	15.87
	8	5	5.55	4.75	0.30	5.10	3.75	16.67
	10	5	5.70	3.50	0.40	5.15	3.25	15.38
Series B	3	3	5.40	7.00	0.07	5.20	6.75	14.81
	4	4	5.40	6.75	0.09	5.10	6.62	15.11
	5	5	5.40	6.75	0.10	5.10	6.50	15.38
	6	6	5.40	7.00	0.13	5.15	6.75	14.81
	7	7	5.45	7.00	0.16	5.10	6.87	14.56
	8	8	5.45	7.00	0.21	5.10	6.75	14.81
Series C	3	0.5	5.35	1.25	0.05	5.10	1.25	13.40
	3	1	5.25	2.50	0.05	5.20	2.25	14.81
	3	2	5.20	4.50	0.00	5.20	4.50	14.81
	3	3	5.10	6.50	0.00	5.10	6.50	15.38
	3	4	5.10	9.00	0.00	5.10	9.00	14.81
	3	5	4.90	11.50	0.00	4.90	11.50	14.49
DISTILLERS' CORN COOKER MASH [2]								
Series A	10	1	6.40	14.00	0.45	5.30	12.50	0.80
	15	1	6.35	9.00	0.60	5.30	8.00	0.83
	20	1	6.30	7.00	0.90	5.25	6.00	0.83
	30	1	6.30	4.50	1.30	5.35	4.00	0.83
	40	1	6.40	3.50	1.70	5.40	3.00	0.83
	50	1	6.40	2.75	2.00	5.25	2.50	0.80
Series B	5	0.25	6.60	8.50	0.30	5.20	6.50	0.77
	10	0.50	6.50	8.00	0.55	5.25	6.00	0.83
	20	1.00	6.30	7.00	0.90	5.25	6.25	0.80
	30	1.50	6.15	7.00	1.20	5.20	6.00	0.83
	40	2.00	6.30	7.00	1.60	5.25	6.00	0.83
	50	2.50	6.15	7.00	2.00	5.30	6.25	0.80
Series C	20	0.2	6.20	2.00	1.00	5.25	1.50	0.67
	20	0.4	6.20	3.00	1.00	5.35	2.50	0.80
	20	0.6	6.30	5.00	1.00	5.25	3.75	0.80
	20	0.8	6.35	6.00	1.10	5.20	5.00	0.80
	20	1.0	6.30	7.00	1.20	5.20	6.25	0.80
	20	1.2	6.00	8.50	1.30	5.20	7.25	0.83

[1] Prepared by extracting 100 g. ground malt with 400 ml. distilled water for one hour at 30°C. A different distillers' malt was used for each series.

[2] The same liquid was used for all three series.

From this equation, it is evident that when the $\frac{\text{starch}}{\text{liquid}}$ ratio is kept constant, the conversion time must be constant for a given enzyme solution.

$$t = \frac{S}{kL} = K. \tag{3}$$

Within certain limits, the t values determined at optimum pH in Series B are constant.

Since reaction velocity varies directly with the enzyme concentrations, the conversion time t will vary directly with the amount of starch (S) when enzyme concentration is constant.

$$t = K_2S. \tag{4}$$

The experimental values for Series C indicate that the changes in conversion time bear a linear relation to changes in the amount of starch.

The data, therefore, demonstrate that the *Converting Capacity* of a given amylase solution determined according to the prescribed method is independent of variations in the volume of enzyme solution or quantity of starch over wide limits and prove the validity of equation (1) on which the determination of the *Converting Capacity* (C) is based.

The accuracy of the method is affected by such factors as error in reading the end point, error in adjusting the pH to the optimum, and the effect of thermal destruction of the enzyme during the conversion. With good technique it is possible to determine t to the nearest 0.25 minute.

Effect of pH. Experiments were conducted to determine the optimum pH for *Converting Capacity* at 62.5°C., and to ascertain the effects of variations in starch and enzyme concentration on the optimum pH.

The conversion times of amylase solutions were determined at pH values from about 3.7 to 8.0 and the *Converting Capacities* calculated. In addition, the enzyme concentration in the digest, volume of enzyme solution × *Converting Capacity* (LC), and the ratio of starch to enzyme volume $\frac{100\ S}{L}$ were calculated. The determinations were grouped in three series:

In the first, both the amount of starch (S) and enzyme solution (L) were kept constant and the pH varied from 3.8 to 8.2.

In the second, the volume of enzyme solution (L) was kept constant (2 ml.) and the starch was varied between 0.5 and 10 g. to give starch to enzyme volume ratios of 500, 250, 125, 50, and

25. With each amount of starch, eight determinations were made at pH values from 4.2 to 5.6, about 0.2 apart.

In the third series, the quantity of starch (S) was kept constant and the volume of enzyme solution (L) was varied (5, 10, 15, and 20 ml.); for each volume, determinations were made at pH values ranging from 4.2 to 6.0.

The results of the first series, summarized in Table II, show that optimum activity of the malt infusion is obtained between about pH 5.0 and 5.4.

The second series of experiments established that changes in the amount of starch (0.5 to 10 g.) with a constant volume of enzyme solution did not change the optimum pH.

TABLE II

EFFECT OF pH ON THE CONVERTING CAPACITY OF MALT INFUSION AT 62.5°C.

pH	Converting Capacity (C)	pH	Converting Capacity (C)	pH	Converting Capacity (C)	pH	Converting Capacity (C)
3.80	0.00	5.00	19.20	5.30	19.30	6.40	9.72
4.10	1.18	5.00	20.00	5.40	19.30	6.75	7.70
4.25	2.73	5.10	20.00	5.45	19.30	6.75	7.28
4.35	13.47	5.15	20.02	5.70	18.13	7.00	5.73
4.55	14.20	5.15	19.30	5.90	14.87	7.20	3.16
4.70	18.13	5.25	19.30	6.10	14.56	7.90	0.94
4.85	18.71	5.30	19.00	6.20	13.20	8.15	0.91

The third series of experiments indicated that there is a slight shift in the optimum pH with a change in the volume of any given enzyme solution. Additional experiments with different solutions indicated that the optimum pH is a function of the enzyme concentration as expressed by the *enzyme volume* × *Converting Capacity* (LC). However, the *Converting Capacity* (C) only changed about 5% at a pH of 5.0 to 5.2 with variations in LC values from 5 to 225.

The optimum pH for different LC values was found to be:

LC	pH
225	4.75–4.95
150	4.85–5.05
75	5.00–5.20
24	5.25–5.45
5	5.65–5.75

The enzyme concentration in the digest should be selected, if possible, to fall within LC values of 50 to 150 when using the pH optimum of 4.9 to 5.2. When log LC is plotted against the optimum pH the result is a straight line satisfying the equation:

$$\text{pH(optimum)} = -0.515 \log LC + 6.06 \qquad (5)$$

Effect of Temperature. The effect of digestion temperature on the *Converting Capacity* of a malt infusion was studied at 12 temperatures between 20° and 80°C. at pH 4.9 to 5.2 Above 60°C. two determinations, and at 70° and 80°C. three determinations, were made with different enzyme concentrations to demonstrate the effect of thermal inactivation on the determination. The data reported in Table III demonstrate that the activity increases up to 62.5°C. Between 30° and 50°C., the activity increases about 1.7 times per 10°C.

TABLE III

INFLUENCE OF DIGESTION TEMPERATURE ON CONVERTING CAPACITY
(pH = 4.9–5.2)

Digestion temp.	Enzyme solution	Starch	Conversion time	Converting Capacity
°C.	*ml.*	*g.*	*min.*	*C*
20.0	30.0	2.5	25.00	0.33
30.0	6.0	5.0	16.00	5.42
40.0	5.0	5.0	10.50	9.52
50.0	4.0	5.0	7.50	16.66
55.0	3.0	5.0	8.00	20.82
60.0	2.0	5.0	10.50	23.80
62.5	2.0	5.0	10.00	25.00
62.5	10.0	5.0	2.00	25.00
65.0	2.0	5.0	10.00	25.00
65.0	10.0	5.0	2.00	25.00
67.5	2.0	5.0	11.50	21.73
67.5	10.0	5.0	2.00	25.00
70.0	2.0	5.0	18.00	13.88
70.0	3.0	5.0	8.50	19.60
70.0	10.0	5.0	2.00	25.00
72.5	2.0	5.0	30.00	8.33
72.5	5.0	5.0	5.00	20.00
75.0	5.0	5.0	25.00	4.00
75.0	10.0	5.0	2.50	20.00
77.5	5.0	5.0	—	0.00
77.5	10.0	5.0	5.00	10.00
80.0	5.0	5.0	—	0.00
80.0	10.0	5.0	32.00	1.56
80.0	20.0	5.0	1.50	16.66

At the optimum temperature of 62.5°C. (and also at 65°C.) the value of *C* is the same for 2 and 10 minutes conversion time so that thermal inactivation was not evident in this length of time, possibly due to the protective activity of substances present in the digest. The first evidence of thermal inactivation is seen at 67.5°C., but even at 70°C. full activity was obtained when the conversion time was decreased to 2 minutes. These experiments demonstrate that no serious thermal destruction occurs at 62.5°C. if the conversion time is maintained between 4 to 8 minutes.

Thermal Destruction of the Enzyme and Its Inhibition. The thermal destruction of the enzyme at 62.5°C. was studied in four series of ex-

periments, where the *Converting Capacities* of four different enzyme solutions were determined after exposing them to a temperature of 62.5°C. for various lengths of time just before adding to the starch solution. The four amylase solutions were: (1) a malt infusion prepared by extracting ground malt with water, (2) a cooker mash, (3) a malt infusion prepared by extracting malt with previously boiled cooker mash, and (4) a malt infusion prepared in a similar fashion to (1) but enriched with 10% maltose.

Before hydrolysis the pH was adjusted to the optimum (4.9 to 5.2) in each case, and the temperature of the amylase solutions was raised to 62.5°C. in less than 2.5 minutes prior to the heat treatment. The

TABLE IV

EFFECT OF TIME ON AMYLASE DESTRUCTION AT 62.5°C. IN VARIOUS EXTRACTS
(pH = 4.9–5.2)

Exposure time	Aqueous malt extract[1]		Cooker mash[2]		Malt extract with cooker mash[3]		Malt extract with maltose added[4]	
	Converting Capacity	Activity	Converting Capacity	Activity	Converting Capacity	Activity	Converting Capacity	Activity
min.	*C*	%	*C*	%	*C*	%	*C*	%
0	13.60	100	1.66	100	24.85	100	12.99	100
5	11.60	84	1.62	97	23.81	96	12.99	100
10	10.35	76	1.55	93	22.86	92	12.42	96
15	9.23	68	1.51	90	19.70	79	12.42	96
30	7.21	53	1.28	77	14.81	60	11.91	92
45	6.10	45	1.13	68	12.50	50	10.58	81
60	5.43	40	1.00	60	11.90	48	10.20	78
90	4.46	33	0.57	34	10.02	40	8.93	69
100	4.35	32	0.41	25	9.15	36	7.54	57

[1] 100 g. ground malt extracted in 400 ml. H_2O at 30°C. Balling = 5.05, pH = 5.85.
[2] Straight cooker mash. Balling = 18.0, pH = 5.30.
[3] Same as [1] except malt was extracted in boiled cooker mash instead of water. Balling = 28.5, pH = 5.50.
[4] Malt infusion prepared as in [1] with 10% added maltose. Balling = 15.13, pH = 5.70.

starch to enzyme ratio was selected to yield a conversion time between 4 and 8 minutes. The conversion times and the activities retained after the heat treatments, expressed in percentage of the starting activities, are given in Table IV. The delay in heat destruction in series (2), (3), and (4), as compared with series (1), is attributed to the protective action of the maltose.

Use of the Converting Capacity in Distillery Control. Measurement of the enzyme activity of diastatic liquids according to the proposed method with its broad scale is particularly useful in distillery control. The enzyme activities of liquids from any production phase can be compared with each other or directly with the original activity of the

TABLE V

CHANGES IN CONVERTING CAPACITY OF COOKER MASH ON THE WAY FROM THE COOKER TO FERMENTER

Converting Capacity in cooker	Time between samples	Converting Capacity in fermenter	Activity lost
C	*min.*	*C*	%
1.25	25	1.19	4.0
0.89	20	0.81	9.0
0.89	20	0.89	0.0
0.96	20	0.74	23.0
1.19	15	1.09	8.0
1.33	20	1.07	19.0
1.27	15	1.18	7.0
1.07	40	0.91	15.0
1.57	30	1.40	10.0

malt which is used. To demonstrate this, *Converting Capacity* for two groups of samples collected from various production phases was determined at the optimum pH (4.9 to 5.2).

In the first group, samples were taken from nine freshly converted cooker mashes coming from the cooker and also from the same mashes after they passed the coolers. The temperature of the mash was 62°C. before entering the cooler and about 25°C. on leaving it. The time which elapsed between each pair of samples, their activity, and the per cent activity lost are given in Table V.

In the second group of samples the loss of amylase activity in beer from the time of inoculation until the end of the fermentation was followed in four separate fermenters. The *Converting Capacities* of samples taken at different intervals and the losses in activity are sum-

TABLE VI

CHANGES IN CONVERTING CAPACITY OF BEER DURING FERMENTATION

Age of beer	Fermenter No. 1		Fermenter No. 2		Fermenter No. 3		Fermenter No. 4	
	Converting Capacity	Loss	Converting Capacity	Loss	Converting Capacity	Loss	Converting Capacity	Loss
hr.	*C*	%	*C*	%	*C*	%	*C*	%
0	1.07	0	1.18	0	0.94	0	1.40	0
5	0.81	24	0.77	35	0.79	16	1.10	22
10	0.71	34	0.75	37	0.54	43	0.70	30
20	0.50	53	0.73	38	0.42	56	0.55	45
24	0.32	70	0.71	40	0.38	60	0.36	64
28	0.21	80	0.63	47	0.21	78	0.31	69
44	0.07	94	0.11	90	0.06	94	0.12	88
48	0.02	98	0.08	93	0.05	95	0.05	95
52	0.02	98	0.06	95	0.04	96	0.04	96
68	0.00	100	0.04	96	0.04	96	0.02	98
72	0.00	100	0.02	98	0.03	97	0.00	100

marized in Table VI. The extensive reduction in amylase activity is the cause of the slow conversion rate during the later phases of fermentation.

The *Converting Capacity* of the beer at any given age indicates the activity of the enzyme under the conditions existing in the fermenter. This may be determined at the pH and temperature of the beer or, in an accelerated test, at the pH of the beer but at 62.5°C. Determinations of the *acting Converting Capacity* parallel with determinations of the *maximum Converting Capacity* reveal that the change in pH which occurs in the fermenter inhibits amylase activity.

In a water mash, after 30 to 34 hours fermentation, the acids produced may decrease the pH to 4.0 or below, at which pH the activity of the amylase is negligible. Because of this, conversion and fermentation cease in spite of the presence of considerable amount of amylase. When stillage is added to the beer, the pH remains at higher values due to the buffering effect, permitting a more complete conversion and fermentation.

In industrial control the determination of both *maximum* and *acting Converting Capacity* has proven to be a useful tool.

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FEEDING TESTS WITH CHLORINE DIOXIDE–TREATED FLOUR [1]

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ABSTRACT

Dogs have been fed flour rations treated with as much as 4 g. chlorine dioxide per hundredweight of flour for as long as 13 weeks and have remained in a healthy condition without any indication of "running fits." Rabbits and monkeys grew well and maintained themselves on chlorine dioxide-treated rations for periods of 6 weeks and 5½ months, respectively. Blood analyses and histological examination of these animals were all within normal limits. Rats grew equally well on untreated or chlorine dioxide-treated flour and bread rations.

Thirteen human subjects were fed normal diets supplemented with large amounts of wheat flour and wheat gluten treated with high levels of chlorine dioxide. Electroencephalograms, neurological examinations, blood analyses, and urinalyses were all within normal limits for each subject at the end of the 6-week experimental periods.

It was first reported by Mellanby (1) that dogs developed "running fits" when fed rations containing wheat flour treated with nitrogen trichloride. Newell *et al.* (2) reported that cats, ferrets, and mink were also susceptible to the nitrogen trichloride toxicity. Monkeys did not develop running fits when fed nitrogen trichloride-treated rations but abnormal brain wave patterns were reported.

In November, 1947, the Food and Nutrition Board of the National Research Council published the following statements concerning the use of nitrogen trichloride: ". . . in view of the susceptibility of several mammalian species there is definite risk of injury to human beings. . . . Alternate processes of flour treatment, especially with chlorine or chlorine dioxide, should be thoroughly explored to make certain that flour so treated is free from toxic effects." With these recommendations in mind, investigation of chlorine dioxide was undertaken. Studies were made on animals known to be susceptible to nitrogen trichloride toxicity and on human beings.

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Materials and Methods

Mongrel dogs ranging in age from 3 to 6 months were used in these experiments. Each animal was dewormed, dusted with DDT (4,4′-dichlorodiphenyltrichloroethane), protected against distemper with injections of canine antidistemper serum, and maintained on a purified basal ration for a week or more before being placed on experiment.

The ration used for the dogs had the following composition: wheat flour, 84; crude casein, 5; corn oil, 5; salts IV, 4; and whole liver powder, 2. Oral supplements of 0.75 mg. thiamine, 0.75 mg. riboflavin, 0.5 mg. pyridoxine, 0.25 mg. pantothenic acid, 1 mg. nicotinic acid, and 70 mg. choline per kilogram of body weight and 6 drops of halibut liver oil were administered twice weekly. Three dogs were fed the above ration containing untreated flour for 12 weeks; two were fed flour treated with 0.5 g. of chlorine dioxide per hundredweight for 6 and 12 weeks; one was fed flour treated with 1.0 g. of chlorine dioxide per hundredweight for 6 weeks; three were fed flour treated with 2 g. of chlorine dioxide per hundredweight for periods of 11 to 13 weeks; two were fed flour treated with 4 g. of chlorine dioxide per hundredweight for 12 weeks. Consumption records were kept for each animal so that an accurate evaluation of the particular flour under test could be made if toxicity developed.

TABLE I

RESULTS OF FEEDING RABBITS RATIONS CONTAINING CHLORINE DIOXIDE–TREATED FLOUR

RATION COMPOSITION (per 100 g.)			
Flour	60 g.	Pyridoxine	1.0 mg.
Gum arabic	12 g.	Nicotinic acid	10.0 mg.
Casein	15 g.	Pantothenic acid	3.0 mg.
Brewers' yeast	5 g.	Inositol	10.0 mg.
Fortified soybean oil [1]	4 g.	p-Aminobenzoic acid	10.0 mg.
Salts IV [2]	4 g.	Biotin	0.04 mg.
Thiamine	1 mg.	Folic acid	0.03 mg.
Riboflavin	1.4 mg.	Choline	300.00 mg.

GROWTH RESULTS

Flour.....	Untreated					4 g. ClO_2/cwt.					
Rabbit	1	2	3	4	5	7	8	9	10	11	12
G. gained in 6 weeks	725	410	895	1085	770	1095	1055	1380	740	1110	1090
Abnormalities	None	None	None	None	None	None	None	None	None	None	None

[1] The following vitamins were dissolved in 4 g. of soybean oil: 12 mg. β-carotene, 0.008 mg. D_3, 0.4 mg. K (Menadione), 120 mg. E.
[2] J. Biol. Chem. **109**: 657 (1935).

Weanling male and female New Zealand White rabbits were obtained from Old Orchard Rabbit Farm, Madison, Wisconsin, and placed on rations containing 60% flour for a period of 6 weeks. The composition of these rations is shown in Table I. Five rabbits were fed rations containing untreated flour, and six others were fed rations containing flour treated with 4 g. of chlorine dioxide per hundredweight. At periodic intervals blood samples were obtained from each animal and the usual analyses (serum protein, red blood cell count, white

TABLE II

RESULTS OF FEEDING MONKEYS RATIONS CONTAINING CHLORINE DIOXIDE-TREATED FLOUR [1]

	Bread made from chlorine dioxide-treated (1 g./cwt.) flour	Bread made from untreated and chlorine dioxide-treated (4 g./cwt.) flour
	%	%
Flour	78	68
Casein	5	15
Corn oil	5	5
Salts IV	4	4
Whole liver powder	2	2
Yeast	3	3
Sucrose	3	3

MAINTENANCE RESULTS

Flour.....	Untreated			1 g. ClO_2/cwt.		4 g. ClO_2/cwt.			
Monkey	500	503	505	C	D	501	502	504	506
Months on expt.	5½	5½	5½	1½	1½	5½	5½	3	3
G. change in weight	+725	+930	+1880	+175	+865	+1880	+2085	−775	+1020
Abnormalities	None	None	None	None	None	None	None	[2]	None

[1] Vitamin supplements per 100 g. of dried, ground bread: 25 mg. ascorbic acid, 1 mg. thiamine, 1 mg. riboflavin, 1 mg. pyridoxine, 5 mg. nicotinic acid, 20 μg. biotin, 3 mg. pantothenic acid, 25 mg. choline, 15 mg. inositol, 50 mg. p-aminobenzoic acid, 100 μg. folic acid. Two drops of halibut liver oil were administered to each monkey weekly.

[2] Cause of weight loss undetermined.

blood cell count, differential white cell count, and hemoglobin) were made. At the end of the test period the animals were sacrificed, examined post mortem, and samples from the central nervous system, all vital organs, and representative tissues were removed and studied histologically.

Nine monkeys were fed bread rations made from untreated flour and chlorine dioxide-treated flour for periods of 6 weeks to 5½ months. The composition of the rations and the results of these tests are given in Table II. Three monkeys were fed untreated bread rations for 5½ months; two monkeys were fed bread made from flour treated with 1 g. of chlorine dioxide per hundredweight for a period of 6 weeks; four

monkeys were fed bread made from flour treated with 4 g. of chlorine dioxide per hundredweight for as long as 5½ months. Blood samples were obtained at 5-week intervals from the monkeys on the long-term tests. The blood was analyzed for serum protein, red blood cell count, white blood cell count, differential white cell count, and hemoglobin. At the end of the test period the animals were sacrificed, examined post mortem, and samples from the central nervous system, all vital organs, and representative tissues were removed and studied histologically.

Weanling male rats of the Sprague-Dawley strain were fed rations containing whole wheat flours, white flours, and bread baked from the

TABLE III

RESULTS OF FEEDING RATS RATIONS CONTAINING CHLORINE DIOXIDE–TREATED FLOUR [1]

Group	I	II	III	IV	V	VI
Flour	Untreated whole wheat flour	1.25 g. chlorine dioxide per cwt. of whole wheat flour	Untreated white flour	4 g. chlorine dioxide per cwt. of white flour	Bread from flour, Group III	Bread from flour, Group IV
	%	%	%	%	%	%
Flour	84	84	81	81	78	78
Casein	5	5	5	5	5	5
Corn oil	5	5	5	5	5	5
Salts IV	4	4	4	4	4	4
Liver powder	2	2	2	2	2	2
Yeast	—	—	—	—	3	3
Sucrose	—	—	3	3	3	3
Rats per group	6	6	20	20	20	20
Avg. wt. at end of 5 weeks (g.)	216	218	174	184	187	177
Abnormalities	None	None	None	None	None	None

[1] Vitamin supplements of: 2 mg. thiamine, 1 mg. riboflavin, 3 mg. pyridoxine, 20 mg. pantothenic acid, 1 g. choline were mixed into each kg. of flour ration and into each kg. of dried, ground bread ration. One drop of halibut liver oil administered orally per rat per week.

white flours for 5-week periods. The whole wheat flours were untreated or treated with 1.25 g. of chlorine dioxide per hundredweight; the white flours were untreated or treated with 4 g. of chlorine dioxide per hundredweight. Composition of the rations fed the rats is shown in Table III.

Thirteen healthy volunteer male and female human beings were fed normal diets supplemented daily with 55 g. of wheat protein treated with high amounts of chlorine dioxide for periods of 6 weeks. These volunteers ranged in age from 19–25 and were students at the university. Each subject received a neurological examination before being

fed the test diet, midway through the experiment, and at the end of the test period. Examination was made of the skull, spine, cranial nerves, motor system, sensory system, autonomic nervous system, blood pressure, and mental status of each subject. Complete blood counts, urinalyses, and serum protein determinations were also made. Electroencephalograms (measurements of brain wave patterns) were taken at 3- to 4-day intervals on all subjects during the test period. Each subject had six to eight control electroencephalograms taken before being started on the test diet. Food supplements were weighed and the net daily consumption of the supplement was tabulated by hospital dieticians. Flour treated with 4 g. of chlorine dioxide per hundredweight and wheat gluten treated with 20 g. of chlorine dioxide per hundredweight were included in daily supplements of cereal and muffins. The wheat products used received four to eight times the average commercial treatment of 0.5 g. of chlorine dioxide per hundredweight of flour, and were fed in considerably higher amounts than are found in the average daily diet.

Results and Discussion

It is evident from the results in Table IV that the three dogs which received untreated flour and the eight dogs which received flour treated with varying levels of chlorine dioxide showed neither running fits nor

TABLE IV

RESULTS OF FEEDING DOGS RATIONS CONTAINING CHLORINE DIOXIDE-TREATED FLOUR

Dog no.	Flour treatment per cwt.	Days on experiment	General condition	Abnormalities
236, 248, 251	Untreated	84	Good	None
241	0.5 g. ClO_2	42	Good	None
246	0.5 g. ClO_2	84	Good	None
27	1.0 g. ClO_2	42	Good	None
250	2.0 g. ClO_2	77	Good	None
30 [1]	2.0 g. ClO_2	84	Good	None
244	2.0 g. ClO_2	91	Good	None
293 [1]	4.0 g. ClO_2	84	Good	None
299 [1]	4.0 g. ClO_2	84	Good	None

[1] Normal electroencephalograms obtained at end of test period.

any other abnormalities which could be attributed to the chlorine dioxide treatment. All animals were in a healthy condition and showed steady weight gains throughout the experiment. Electroencephalograms were taken on dogs 30, 293, and 299 at the beginning and at the end of the test period. Although flour treatment was at the high level of 4 g. of chlorine dioxide per hundredweight and the test ration was fed for 84 days (12 weeks), no abnormalities were observed

in the electroencephalograms of any of the dogs. Previous experience, Newell *et al.* (2), indicated that flour treated with low commercial levels of nitrogen trichloride (1 g./cwt.), and fed at high levels of the ration, produced running fits in dogs in at least 45 days. It was felt that any toxic condition which might develop in dogs as a result of chlorine dioxide treatment of flour would occur within a 12-week period.

Growth results obtained with rabbits fed rations containing 60% of flour for 6 weeks are summarized in Table I. The semipurified rations fed these animals sustained good growth when compared with natural rations fed rabbits in this laboratory. Rabbits fed nitrogen trichloride-treated flour rations (20 g./cwt.) readily develop running fits in 5 to 10 days and die or are permanently paralyzed. Rabbits fed the untreated or chlorine dioxide-treated flour were in a healthy condition throughout the experiment. Those fed the treated flour rations grew better than those fed the untreated flour rations, but no significance was attributed to this difference. Blood analyses and histological examination of the tissue samples were all within normal limits.

Results obtained with monkeys fed bread made from untreated and chlorine dioxide-treated flour are summarized in Table II. One animal suspected of having incipient tuberculosis was sacrificed after being on experiment 3 months. However, post mortem examination of this animal showed no abnormal conditions and histological examination of the tissues revealed no abnormalities. All other monkeys used in this work grew and maintained themselves throughout the test period. Blood analyses were normal; post mortem examinations showed no pathological conditions; histological examination of the tissues revealed no abnormalities.

Growth results of rats fed flour and bread rations are shown in Table III. Rats fed the untreated and chlorine dioxide-treated whole wheat rations grew well during the 5-week test period. Rats fed flour or bread rations made from treated white flour showed growth responses similar to those of rats fed flour or bread rations made from untreated white flour. There was no evidence of toxicity from any of the flours, even when the 4 g. of chlorine dioxide per hundredweight treatment was used.

All subjects fed the chlorine dioxide-treated food supplements for 6-week periods remained in healthy condition throughout the experiment. Electroencephalograms were normal for each subject and showed no changes from the normal patterns obtained during the control period. Most subjects gained weight while on the test diet as a result of the high protein intake. Blood, urine, and neurological

examinations made on each patient were normal at the end of the experiment.

Acknowledgments

We are indebted to Dr. H. K. Parker, to Meade C. Harris, and to Karl L. Fortmann, of the Wallace and Tiernan Company, for preparing the chlorine dioxide-treated food materials.

We wish to thank Dr. J. A. Dingwall III, of Cornell Medical College, New York City, for making histological examinations of the rabbit and monkey tissue samples; Miss Ruth Dickie and Mrs. Ruth Balliette for their help in preparing diets for the human subjects; Miss Phyllis Helmer and Miss Hulda Gieschen for their assistance in recording the electroencephalograms.

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HYGROSCOPIC EQUILIBRIA OF WHOLE KERNEL CORN [1]

S. F. BROCKINGTON,[2] H. C. DORIN,[2] and H. K. HOWERTON [3]

ABSTRACT

The relative humidities of air in equilibrium with shelled yellow corn conditioned to moisture contents between 9.4 and 22.6% were determined at 80° ± 0.2°F. with a new type of electric hygrometer. Moisture determinations were carried out with the Brown-Duvel apparatus as well as by a two-stage vacuum oven technique in which the second stage involved drying at 135°C. in a vacuum oven for 5 hours. The critical moisture content of shelled, yellow corn for safe storage, namely that in equilibrium with a relative humidity of 75%, was found to be 13.8 ± 0.2% by the Brown-Duvel method and 14.7 ± 0.1% by the two-stage vacuum oven technique.

It is well recognized that spoilage in stored grain depends largely on its moisture content. Above a certain critical moisture range, marked acceleration in the respiratory rate occurs and heating tendencies appear. Milner and Geddes (6), Gilman and Semeniuk (5), and others have shown that this acceleration of respiration and the accompanying increase in fat acidity of various grains occurs at moisture contents in equilibrium with a relative humidity of approximately 75%, which is about the lowest humidity at which the common storage molds (species of *Aspergillus* and *Penicillium*) present on and in the grain will grow. It is therefore of prime importance in the safe storage of corn to know the moisture content at which the grain is in equilibrium with 75% relative humidity.

[1] Manuscript received October 6, 1948.
[2] The Quaker Oats Company, Research Laboratories, Chicago, Illinois.
[3] The American Instrument Company, Silver Spring, Maryland.

Many determinations of the hygroscopic equilibria of various grains have been made. Coleman and Fellows (2) passed air at various humidities over previously dried grain and determined the moisture contents after equilibrium was attained. Their moisture data were computed on the dry basis and must be multiplied by the factor 100/100 + M, where M is the moisture content on a dry basis to convert them to the customary values on the wet basis. Oxley (7) has recently discussed hygroscopicity data obtained on cereal grains by several workers. Although there is lack of agreement concerning the equilibrium moisture values for given humidities, all investigators obtained curves of the sigmoid type. The relative humidity increases rather sharply with increases in moisture between 4% and 16% and small increases in moisture will therefore cause wide increases in ambient relative humidity. Cereal grains containing between 14% and 15% moisture are in approximate equilibrium with 75% relative humidity. Above 15% moisture, the humidity increases more slowly with increase in moisture and the curves tend to approach an asymptotic value at about 95% relative humidity.

Oxley (7) suggests that it would be better to measure ambient humidity of the surrounding air in grain directly, after previously wetting, rather than to pass air at different humidities over dried grain in order to study hygroscopic equilibria. The sigmoid shape of the curves suggests that the absorption of water by grain and vapor pressure of moist grain follow hysteresis patterns.

The present study is concerned with the determination of the ambient relative humidity of whole kernel yellow corn, conditioned to various moisture contents, by an electric hygrometer similar to that described by Dunmore (4). This instrument was found to be extremely sensitive and afforded a relatively simple method for determining the relative humidity in a small space.

Materials and Methods

Apparatus. The electric hygrometer, consisting essentially of a humidity-sensing element and a microammeter with necessary electrical connections, was made and calibrated by the American Instrument Company. The composite sensing element, hereafter designated 4TH2, was constructed from four single elements. The operation of the element is based on the ability of a hygroscopic film to change its electrical resistance quickly with small changes in moisture content of the air surrounding it. The resistance was measured in microamperes of alternating current flowing through the element as shown by the electrical indicator. A single element is shown in Fig. 1.

By electrically coupling four single elements through various

resistors, the completed 4TH2 element when connected to the indicator gave readings which were proportional to the relative humidity. A small resistance thermometer was incorporated in the 4TH2 element as a temperature-measuring device to determine the necessary corrections in humidity readings for variations in temperature. The instrument was calibrated approximately one week before the experiments on corn were made, in a controlled atmosphere. Wet- and

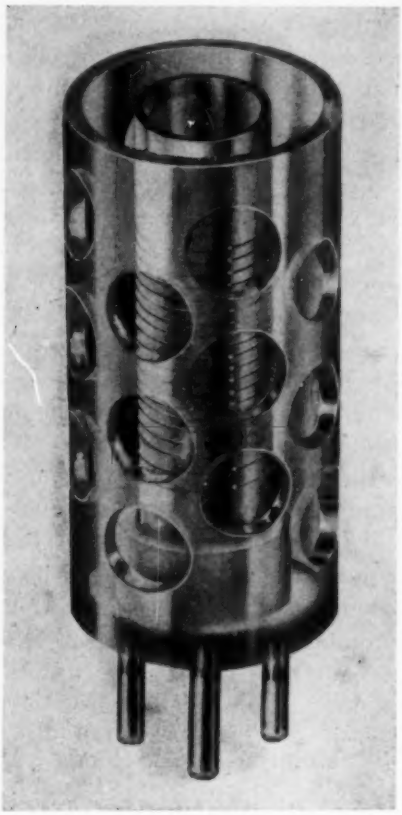

Fig. 1. Type H-2 humidity-sensing element.

dry-bulb temperatures were measured by mercury-in-glass thermometers to an accuracy of ±0.5°F. Depression of the wet bulb for various humidities was extrapolated from the psychrometric tables published by the Weather Bureau, U. S. Department of Commerce. The calibration curves for three temperatures are shown in Fig. 2 and are accurate to within 0.5% relative humidity.

The corn was placed in a quart jar as shown in Fig. 3. The 4TH2 element was mounted on the cover of the jar by means of an amphenol

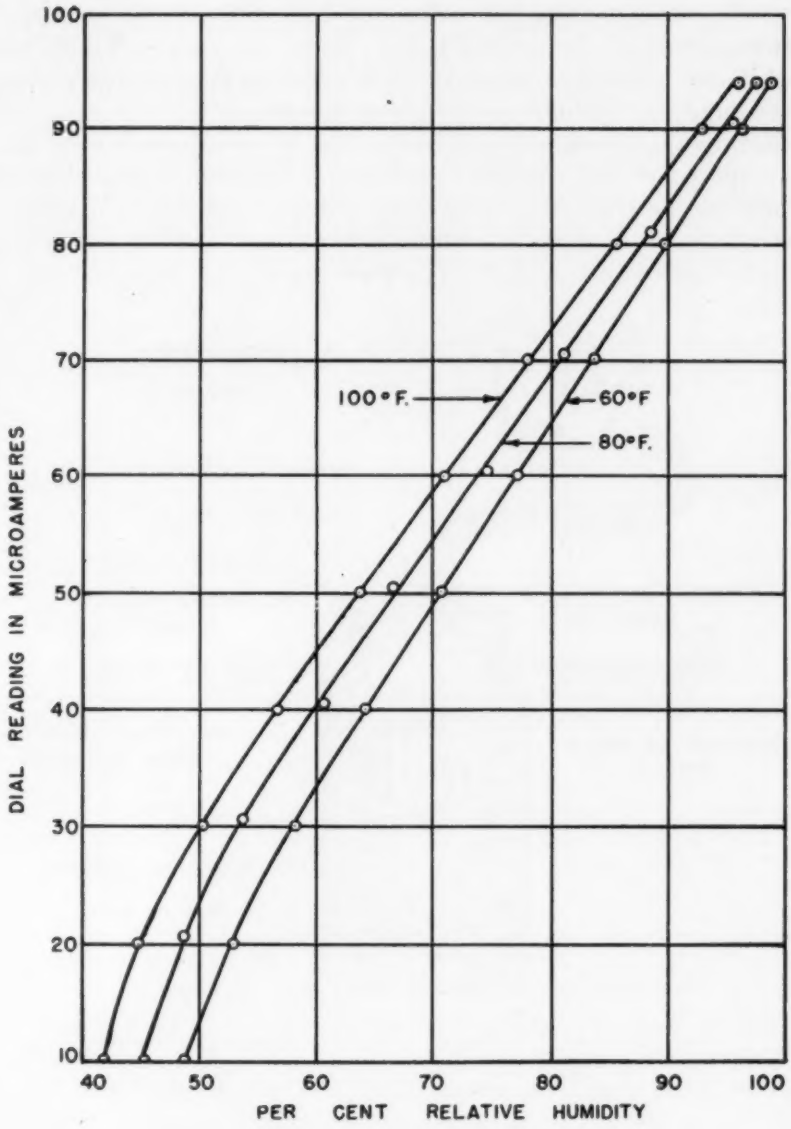

Fig. 2. Calibration of humidity-sensing element.

connector which served as a receptacle for the leads and as a means of obtaining an effective seal between the cover and the element. A fine mesh brass screen was placed around the element for protection.

The corn used in these experiments was naturally dry No. 2 yellow corn of the 1947 crop which was approximately 7 months old. It was cleaned free of debris and broken kernels in the laboratory.

Sufficient tap water was added to 1-kg. samples to increase the water content to the desired value. The samples were mixed thoroughly and allowed to stand 16 to 24 hours to equalize the moisture throughout, before humidity determinations were made.

Humidity Determination. About 500 g. of the moistened grain were placed in the container, the sensing element completely submerged in the grain, the cover screwed down, and the jar placed in a water bath at 80° ± 0.2°F., with the water level in relation to the jar adjusted as shown in Fig. 3. The kernels were thus kept in close con-

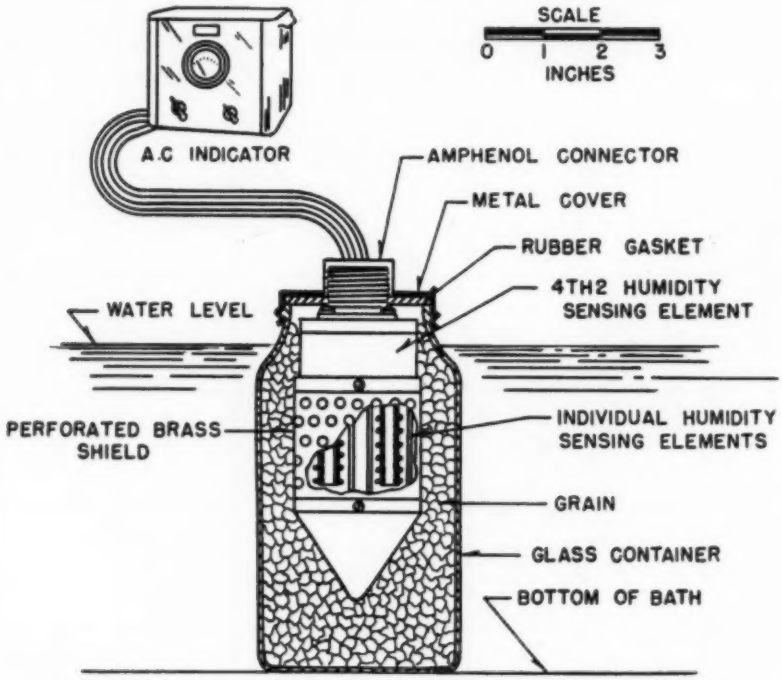

Fig. 3. Diagram showing assembly of apparatus for measuring the equilibrium relative humidity of corn of various moisture contents at constant temperature.

tact with the protective screen which did not interfere with the distribution of the air in contacting the sensitive hygroscopic film of the element, yet kept the grain from making direct contact. Various attempts had previously been made to circulate the air within a container and around the element by using a small turbine-type stirrer dipping into the grain. However, it was impossible to secure consistent humidity readings due to the large volume of air that had to be used in proportion to the small volume of corn, and equilibrium conditions were difficult to obtain. It was found that by keeping the ratio of air to corn low and measuring humidity of the air under static

conditions, more accurate readings could be obtained. Practically no air space was present between the cover and the top of the grain in the jar. Humidity readings were taken at intervals of 15 minutes until no further change in humidity was observed. The temperature and humidity reached equilibrium in about 2 hours.

Moisture Determinations. Brown-Duvel moisture determinations were made by a strictly standardized procedure which involved electrically heating a 100-g. sample of corn in 150 ml. of special moisture tester oil to 190°C. for 20 minutes, allowing to cool to 160°C., and reading the volume of water which had distilled. Single determinations were made by this method, but repeated tests showed that the duplicate determinations did not vary by more than 0.2%.

Duplicate moisture determinations were made by a two-stage vacuum oven procedure (1). The first stage involved drying a 20-g. sample of the whole grain for 0.5 hour in a vacuum oven at 100°C. which reduced the moisture content of all samples below 12%. In the second a 2.0-g. sample of the dried grain, after grinding to pass a sieve with openings 1.0 mm. in diameter, was heated in a vacuum oven at 135°C. for 5 hours.

Results and Discussion

The results are recorded in Table I and are represented graphically in Figs. 4 and 5. The empirical nature of moisture determinations in biological materials is widely recognized. Cook, Hopkins, and Geddes (3) have shown that several methods in common use for grain vary in precision and give different moisture values. They found that, when conducted according to a carefully standardized procedure, the rapid Brown-Duvel method widely used in the grain trade gives reproducible results. In the present study the maximum difference between duplicates was 0.4% by the Brown-Duvel method and 0.2% by the two-stage vacuum-oven method.

The Brown-Duvel method gave lower moisture values than the vacuum-oven method below 17% moisture, whereas the reverse is the case at moistures exceeding this.

Figs. 4 and 5 show that in the region between 12% and 20% moisture, humidity increased rapidly with moisture. At 22% moisture by either method the humidity curve levels off and there is very little change with increased moisture. The ambient humidity tends to approach an asymptotic value at 93% relative. These findings are very similar to results of other workers cited by Oxley (7).

Clean yellow corn of average milling grade at 13.8 ± 0.2% moisture by the Brown-Duvel method, or 14.7 ± 0.1% by the two-stage vacuum oven method, is in hygroscopic equilibrium with the ambient air at 75% relative humidity and a dry-bulb temperature of 80° ±

TABLE I

Relation Between Moisture Content and Interstitial Relative Humidity in Whole Kernel Corn

Sample number	Moisture content		Equilibrium relative humidity	Temp.[2]
	Brown-Duvel	Two-stage oven		
	%	%	%	°F.
1[1]	7.0	9.4	40.0	—
2[1]	7.5	9.9	40.0	—
3	11.6	12.9	59.0	80
4	11.8	12.6	63.5	79
5	12.9	14.1	71.5	80
6	13.2	14.7	71.0	80
7	14.0	15.2	76.5	80
8	14.2	14.6	75.5	80
9	14.6	15.2	79.0	79
10	14.8	15.8	79.0	81
11	15.7	15.8	82.0	78
12	17.3	17.2	85.5	80
13	17.4	17.0	85.0	80
14	17.8	18.2	87.5	81
15	18.0	18.5	86.5	83
16	18.8	18.2	88.0	78
17	18.9	19.4	90.0	79
18	20.0	20.3	90.5	82
19	20.7	19.9	88.5	83
20	22.4	19.5	89.5	83
21	22.5	19.8	91.0	81
22	26.8	22.6	92.0	83

[1] Untempered corn.
[2] Indicated by resistance thermometer.

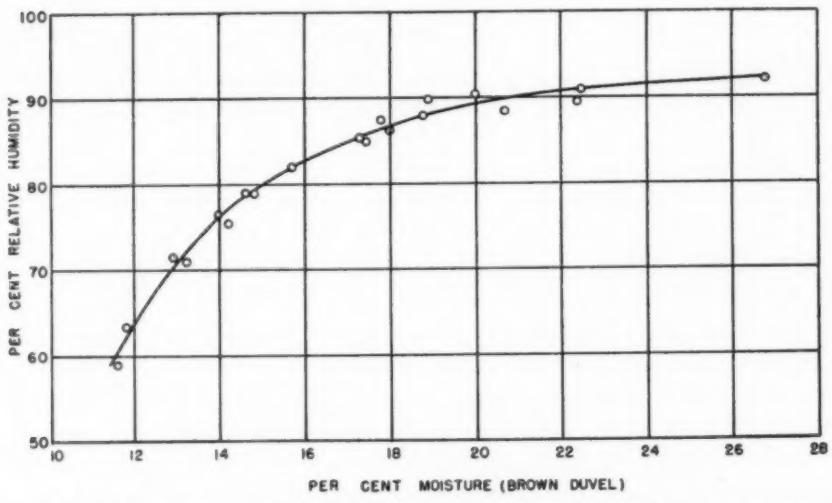

Fig. 4. Relation between relative humidity of interseed atmosphere at 80°F. and the moisture content of shelled whole yellow corn as determined by the Brown-Duvel method. The moisture values are expressed on the wet basis.

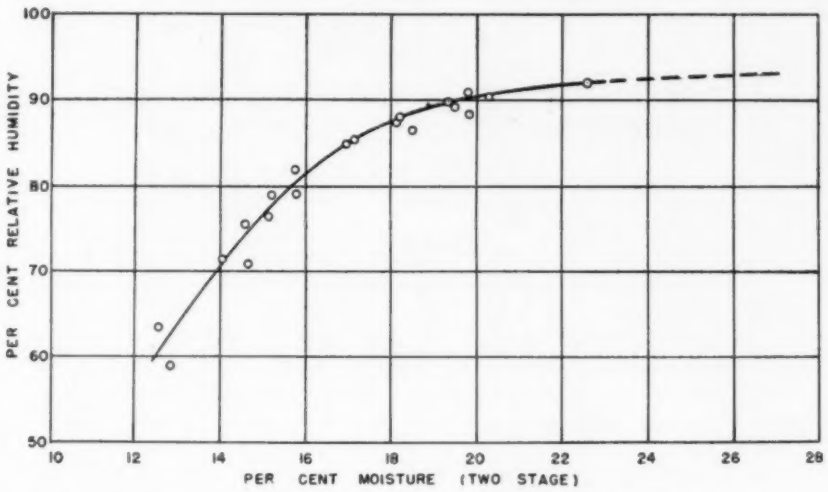

Fig. 5. Relation between relative humidity of interseed atmosphere at 80°F. and the moisture content of shelled whole yellow corn as determined by a two-stage vacuum oven method. The second stage involved heating the ground sample of the previously dried corn for 5 hours at 135°C. The moisture values are expressed on the wet basis.

0.2°F.[4] As the common molds on grain will not proliferate at humidities below 75%, the moisture content of the grain represented by the above values is the maximum which can be regarded as safe for the storage of corn.

The authors consider that the electric hygrometer employed in this study is the best available method for measuring relative humidity under static conditions or in slowly moving air. Equilibrium readings were more rapidly attained than with other techniques heretofore used and their replicability was very satisfactory.

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[4] The ± deviations in moisture and temperature are based on maximum differences between duplicate determinations.

DETERMINATION OF DRY SUBSTANCE IN CONCENTRATED CORN STEEP WATER [1]

BARRETT L. SCALLET and OTTO EBLE [2]

ABSTRACT

In a critical study of the benzene distillation method for determination of dry substance in corn steep water it has been shown that the small amounts of acid which distil over with the water do not affect the results. Continuous slow decomposition is believed to take place in the sample during distillation. Water added to dry starch to give a sample containing 60% moisture requires more than 27 hours for complete distillation into a 20 ml. trap. In general, the rate of removal of known quantities of water from dried, remoistened starch is directly proportional to the moisture content. The nearly constant moisture readings obtained with steep water at 20 to 27 hours are thus considered to approach the true moisture values.

The method has been applied to three samples of viscous concentrated steep water obtained at intervals of about one month. The relationships between dry substance values and densities of the diluted solutions were very similar for all three samples.

For a number of years the determination of dry substance in concentrated corn steep water has been a controversial problem. The principal difficulty in determinations is due to the marked instability of the material towards heat. Early determinations of dry substance by drying at atmospheric pressure at 100°C. gave relatively low results because a considerable amount of the dry substance was volatilized. Later determinations at temperatures of 40°–50°C. under vacuum gave higher results, but no definite conclusions about the accuracy of the method could be drawn.

The application of solvent distillation methods to other corn products by Sair and Fetzer (3) indicated the possibility that the true dry substance of steep water can be determined by these methods. The experiments reported here were undertaken to determine whether the benzene distillation method can be used to obtain a value close to the correct dry substance value of steep water.

Materials and Methods

The benzene distillation method of Cleland and Fetzer (2) was used. Samples of steep water were dispersed on dry filtercel, benzene was added, and the flasks were connected to reflux condensers. The standard-taper joints were sealed with Parafilm wax. Leakage was so

[1] Manuscript received December 1, 1947.
[2] Anheuser-Busch, Inc., St. Louis 18, Missouri.

low by this method that distillation could be continued for 10 days without noticeable loss of benzene. Readings of the water in the traps were made by surrounding the bulbs and graduated portions of the traps with water at 20°C. Readings were taken after 10 minutes, since longer cooling (up to 90 minutes) did not alter the results.

In certain experiments the steep water was neutralized to various pH values with sodium hydroxide solution. For these, a strong sodium hydroxide solution was made up and analyzed for solid content by titration with standard acid. Measured amounts of the caustic solution (about 3–10 ml.) were then added to weighed amounts of steep water (about 150 g.) until the desired pH values were attained (glass electrode). The samples were than analyzed for moisture by benzene distillation.

For the experiments involving neutralization with ammonia, anhydrous ammonia from a cylinder was used. It was passed through two 10-inch drying towers packed with soda-lime and then through a rubber Thomas valve immersed in the weighed steep water. The flow was regulated with a needle valve so that no ammonia escaped into the air. The pH of the steep water was read continuously and the flow of ammonia was stopped when the desired pH was reached. The weight of ammonia added was determined by reweighing the steep water. Moistures were then determined by benzene distillation.

For the experiments in which starch was dried, remoistened, and distilled with benzene, a pearl starch of pH 6.3 was used. Quantities of 36 to 72 g. were weighed out in Erlenmeyer flasks containing a bottom mat of asbestos fiber. The flasks and contents were then dried to constant weight in the Weber oven at 100°C. and 5 mm. of mercury pressure. Total drying time was 48 hours (three 16-hour periods; weight constant after 32 hours). About 19 ml. of water were added to each flask, in such a way that the starch was exposed to the atmosphere for only about 5 seconds; the weight was redetermined, and the water then distilled with benzene.

Traps were calibrated only at the highest graduation. However, sample sizes were selected so that the final readings were very close to the top graduation.

For the series of steep water dilutions, a sample for which the dry substance value had been determined by benzene distillation was diluted progressively with water. A weighed sample of steep water was diluted quantitatively by adding water and reweighing. It was then cooled to 60°F. and the Be. reading determined. The sample was returned to the original beaker, reweighed, and diluted further in the same manner. The range covered was 3°–22° Be.

Results and Discussion

Effect of pH of Sample on Moisture Recovery. In applying the standardized benzene distillation method of Cleland and Fetzer (1), preliminary results with 5 ml. traps indicated that there was a period of near constancy from 3 to 9 hours after the start of distillation. However, the water recovered in the distillate was definitely acid and required several ml. of 0.1 *N* sodium hydroxide to neutralize it to a phenolphthalein end point. This indicated that some of the acids of the steep water were volatile with benzene-water.

Although it was suspected that the influence of this amount of acid on the volume of water recovered was negligible, this point was tested by neutralizing a sample of steep water with sodium hydroxide solution to various pH values before distillation. Since water was added with the sodium hydroxide and water was formed in the neutral-

TABLE I

EFFECT OF pH OF SAMPLE ON RECOVERY OF MOISTURE FROM STEEP WATER

	pH					
	3.6	5	6	7	8	9
Dry substance, % (duplicates)	— 46.2	48.0 49.4	47.8 47.5	49.3 48.4	47.8 48.3	48.6 48.4
Ml. 0.1 *N* sodium hydroxide to neutralize distillate	0.90	0.10	0.05	—	—	—
Ml. 0.1 *N* sulfuric acid to neutralize distillate	—	—	—	0.10	0.12	0.14

ization of acids with the base, a rather involved calculation was necessary to bring all the results to the original steep water basis (Table I).

The results showed that the distillate would be neutral at a sample pH of about 6.3. At higher pH values ammonia was evolved in sufficient quantities to make the distillates alkaline. The dry substance results were too erratic to permit definite conclusions about the pH effect. In this experiment 5 ml. traps were used and the sample weight was about 6 g. The uncertainty in trap readings was about ± 0.02 ml. and an error of 0.01 ml. corresponded to a dry substance error of 0.3%. To increase the precision of the method, 20 and 35 ml. traps were used. The uncertainty of readings was also ± 0.02 ml. for these traps, but much larger samples were used. With a 20 ml. trap and a sample weight of about 40 g., the ultimate precision of the

method should be about 0.1%. The 35 ml. traps should give 0.06% precision.

In actual operation on steep water the precisions obtained were about 1% for 5 ml. traps, 0.4% for 20 ml. traps, and 0.6% for 35 ml. traps.

The moisture contents of neutralized and unneutralized samples of steep water were next determined side by side in 5, 20, and 35 ml. traps. To eliminate difficult calculations and simplify experimental procedure, anhydrous ammonia was used to neutralize half of the

TABLE II

EFFECTS OF NEUTRALIZATION WITH AMMONIA AND OF TRAP SIZE ON RECOVERY OF WATER

Distillation time	Moisture content					
	Trap size, ml.					
	5		20		35	
	pH					
	3.6	6.4	3.6	6.4	3.6	6.4
Hour	%	%	%	%	%	%
1.5	70.77	70.61	67.49	70.71	—	—
6	71.72	71.62	71.31	71.37	71.07	70.84
8	71.72	71.77	71.36	71.56	71.16	70.88
21	72.20	72.20	71.80	71.94	71.63	71.58
28	72.36	72.20	71.94	72.08	71.79	71.64
48	—	—	—	72.12	72.01	71.77
68	72.52	72.35	—	—	—	—
93	72.67	72.64	72.29	72.40	72.31	72.01
Ml. 0.1 *N* sodium hydroxide to neutralize distillate	1.17	0.00	2.00	0.00	4.88	—
Ml. 0.1 *N* sulfuric acid to neutralize distillate	—	—	—	—	—	15.1

sample of heavy steep water to pH 6.4. The results (Table II) show that the pH of the steep water has no recognizable effect on the determination of dry substance. The variations between columns are of the same order as the experimental error. The quantity of acid distilled from the samples at pH 3.6 was not enough to affect the trap readings. The moisture value attained with the 35 ml. traps in 48 hours was attained in 21 hours with the 20 ml. traps and in 8 hours with the 5 ml. traps. However, no period of constant readings was found with any of the samples.

Rate and Completeness of Moisture Removal. Because the periods between readings were long in the previous experiment, any possible plateau in the water-removal curve would probably have been missed. Since there were indications that a plateau might exist at about 20

TABLE III

RATE OF MOISTURE REMOVAL DURING BENZENE DISTILLATION OF FOUR SAMPLES OF STEEP WATER OF DIFFERENT MOISTURE CONTENTS [1]

Distillation time	Moisture collected			
	Sample A[1]	Sample B[1]	Sample C[1]	Sample D[1]
Hours	*ml.*	*ml.*	*ml.*	*ml.*
1.5	17.03	18.16	15.88	15.00
1.75	17.32	18.39	17.09	16.22
2	17.36	18.46	17.47	16.54
2.6	17.43	18.52	17.73	16.87
4.2	17.51	18.64	17.84	16.99
5.2	17.54	18.70	17.93	17.02
13	17.61	18.78	17.98	17.16
14	17.62	18.79	17.99	17.17
15.5	17.64	18.81	18.02	17.20
16	17.65	18.83	18.04	17.20
17	17.66	18.84	18.04	17.20
18	17.66	18.84	18.04	17.23
19	17.66	18.84	18.06	17.22
20.5	17.69	18.87	18.06	17.24
21	17.68	18.86	18.06	17.24
22	17.69	18.86	18.07	17.25
23	17.68	18.87	18.06	17.25
24	17.69	18.88	18.07	17.26
25	17.69	18.88	18.07	17.27
26	17.69	18.88	18.08	17.26
27	17.69	18.88	18.08	17.26
40	17.73	18.93	18.13	17.33
41	17.73	18.92	18.12	17.33
42	17.73	18.92	18.12	17.33
66.5	17.75	18.95	18.15	17.36
90.5	17.77	18.96	18.18	17.38
115	17.79	18.97	18.21	17.39
164	17.82	19.00	18.23	17.43

[1] Densities of the four steep waters after diluting with equal weights of water and weights of the samples taken for distillation were:

Sample No.	°Be.	Sample weight *g.*
A	12.15	20.816
B	13.05	23.726
C	14.50	23.486
D	16.10	23.309

hours with the 20 ml. traps, another experiment was performed (Table III) in which trap readings were taken at hourly intervals from 13 to 27 hours after the start of distillation. The samples were taken at successive stages during the concentration of a batch of steep water.

The results showed only a gradual removal of water, with no extended period of constancy of readings that could be attributed to

completion of removal of water before decomposition set in. It seemed quite probable that a slow decomposition was occurring throughout the distillation, and that the nearly constant readings from 20 to 27 hours gave values close to the true moisture contents of the samples.

To test this point another experiment was performed to find the length of time necessary to remove a known amount of water completely by benzene distillation. An organic material stable to heat and capable of holding water at least as tenaciously as steep water was needed, and corn starch was judged to meet these requirements. Samples of pearl starch of pH 6.3 were dried to constant weight at 100°C. under high vacuum. They were then remoistened with

TABLE IV

RATE OF REMOVAL OF WATER FROM STARCH OF KNOWN MOISTURE CONTENTS

Distillation time	Percentage recovery of water from starch of various moisture contents[1]			
	30%	40%	50%	60%
Hours	%	%	%	%
4	—	—	—	97.77
5.5	—	—	—	99.04
7.5	—	—	—	99.41
10	92.15	—	98.13	—
16	94.79	—	99.20	—
21	95.85	98.54	99.09	—
27	—	—	—	99.67
41	98.07	99.81	100.05	—
69	99.13	100.23	100.21	—
113	99.60	100.50	100.26	—
210	99.92	100.55	100.31	—

[1] Weighed amounts of water were added to pearl starch (dried to constant weight at 100°C. under vacuum) to give the moisture values indicated. Sample weights for the distillation were so selected that 19 g. of water were present in each case.

weighed amounts of water and subjected to benzene distillation. Sample weights were selected so that the addition of 19 g. of water to each would yield wet starches of 30, 40, 50, and 60% moisture contents.

The results (Table IV) show that moisture removal is dependent upon the amount of dry substance present tending to retain it. The greater the amount of starch present, the slower is the removal of water. All of the added water was removed from the starch containing 60, 50, 40, and 30% moisture in the following respective times: 27, 41, 41–69, and 210 hours. Water removed in excess of that added may have been due to slight decomposition of the starch.

Application of these results to the determination of moisture in steep water shows that the observed behavior of steep water during

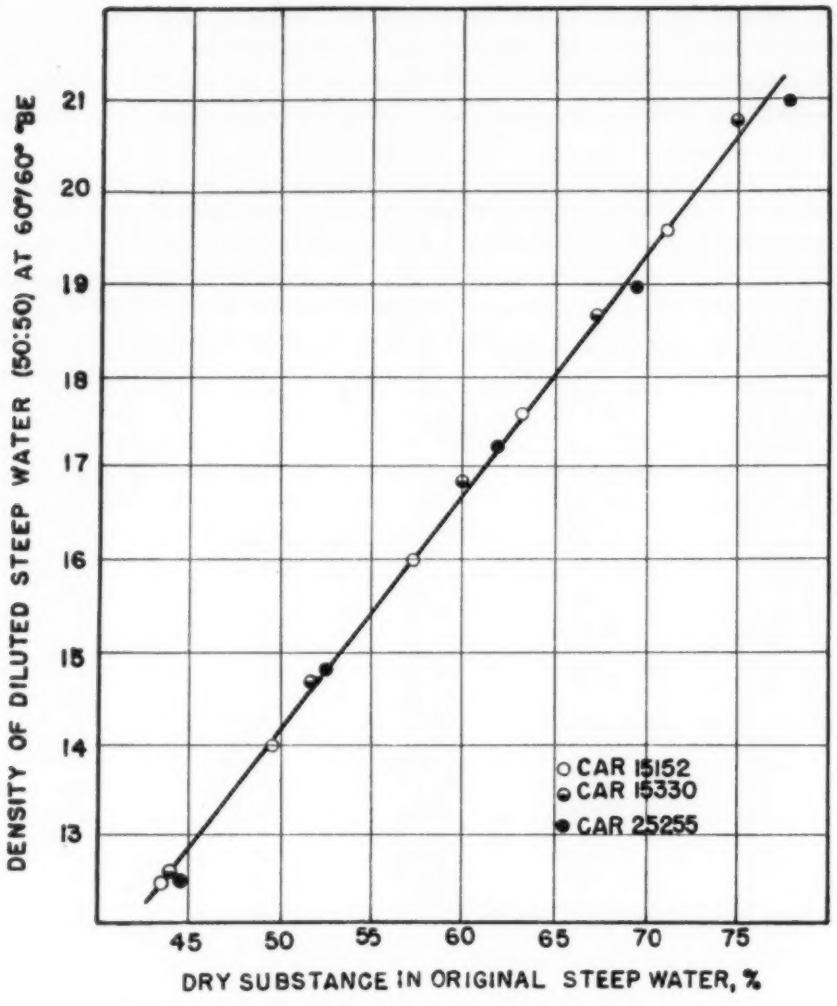

Fig. 1. Curve relating dry substance and Baumé of 50:50 (by weight) dilution of heavy steep water samples.

distillation can be explained by the same sort of slow decomposition. It is thus highly probable that the period of near constancy of moisture readings from 20 to 27 hours (with 20 ml. traps) for steep water of about 70% moisture content gives a value very near to the true moisture value of the steep water.

Application of the Method. The method of determination of dry substance by means of hydrometer readings is generally rapid, reproducible, and relatively accurate. The readings must of course be related to the dry substance by some absolute method such as benzene distillation.

In the case of many concentrated steep waters the hydrometer method is difficult to apply because of the viscous, semicrystalline state of the material.

However, the hydrometer method can be applied readily to diluted samples of this type of steep water. A dilution of 50:50 by weight was chosen for the following reasons:

(a) The physical nature of the 50:50 dilution is such that an accurate Be. reading can be obtained easily and quickly.

(b) A 50:50 dilution rather than a greater or smaller dilution reduces the effect of weighing errors to a minimum.

(c) A dilution by weight rather than volume is more accurate for the semisolid material.

(d) The per cent dry substance value for the dilution is exactly half of the value for the original solution, which simplifies calculations.

Dry substances (determined by benzene distillation) of several steep water samples are plotted against the densities of 50:50 dilutions in Fig. 1. The points fall on a reasonably straight line in this Be. range. This curve applies only to incubated steep water (dextrose converted to lactic acid with Lactobacillus) which has a Be.-dry substance relationship considerably different from that of the nonincubated type. Experience with samples from other companies indicates that steep water varies from company to company and from time to time for a given company. The curve shown was obtained with samples from tank cars which represented a number of batches. The three samples were obtained at intervals of about a month during the fall and show that the steep water is reasonably constant during a season. The relationship should be verified perhaps as often as four times a year.

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SOLUBILITY STUDIES ON THE NITROGENOUS CONSTITUENTS OF SPENT BREWERS' GRAINS [1]

BRUNO VASSEL, S. J. STEPHENS, and P. R. CELUSTA-HOWARD

ABSTRACT

The proteins in dried spent brewers' grains are poorly soluble in the common protein solvents. Less than 10% of the grain nitrogen is extractable with water, 10% aqueous sodium chloride, 0.075 *N* hydrochloric acid, or 0.01 *N* sodium hydroxide. After ball mill grinding of the grains, prolonging the extraction time to several hours, and using 0.5 *N* sodium hydroxide as the solvent, up to 90% of the grain nitrogen can be peptized. However, these conditions also favor the dispersion of the nonnitrogenous constituents of the grains. The net effect is only an insignificant increase of the nitrogen content in the isolated product over that of the starting material. About 75% of the dispersed nitrogen is precipitable at either pH 3.75 or pH 4.55, and about 85% in the presence of 30% of either sodium sulfate or magnesium sulfate ($\cdot 7\ H_2O$) at pH 4.1. The isolated material contains, on an ash and moisture free basis, 69.3% carbohydrates, 4.75% nitrogen, no cystine, 0.65% methionine, and 8.55% glutamic acid.

In the terminology of the brewing industry, spent brewers' grains comprise the residue of the barley malt after germination, removal of the sprouts, mashing, and drawing off of the mash liquor (the wort) from the solids (the spent brewers' grains). The wet spent grains are customarily first pressed, which reduces the moisture from 80% to 65–70%, then dried either by passing hot air through them, or by exposing them to rotating peripheral steam coils or chests (4). The moisture is thereby reduced to below 10%. The dried grains are sold as animal feeds. During 1940–41, 106,000 tons of dried spent brewers' grains reached the market; the amount increased to 230,700 tons in 1943–44, but dropped slightly to 211,900 tons during 1945–46 (1). Since the average crude protein content of the grains amounts to slightly over 25%, it seemed of interest to investigate the feasibility of recovering the proteins for use in industrial processes.

Experimental and Results

The dried spent brewers' grains used in this investigation were taken from a typical commercial lot. They contained 5.3% moisture, 2.6% ash, 25.5% protein (4.13% N [2] × 6.25), and 6.25% ether extractables.[2]

[1] Manuscript received December 6, 1948.
From the research laboratories of the Amino Products Division of International Minerals and Chemical Corporation, Rossford, Ohio.
[2] All analytical results throughout this report are calculated on an ash and moisture free basis.

TABLE I

PEPTIZATION OF SPENT BREWERS' GRAIN NITROGEN DURING ONE-HOUR EXTRACTION PERIODS

Solvent used	pH after shaking	Meal nitrogen extracted
		%
90% (conc.) Formic acid	0.0	34.1
Glacial acetic acid	1.0	17.7
0.075 *N* Hydrochloric acid	1.7	4.3
10% Sodium chloride	5.4	3.1
Water	5.5	2.9
0.01 *N* Sodium hydroxide	9.2	8.1
0.1 *N* Sodium hydroxide	11.7	35.2
0.5 *N* Sodium hydroxide	11.6	52.0
1.0 *N* Sodium hydroxide	11.4	51.9

Protein extractions were performed in 250 ml. centrifuge bottles. One hundred ml. of solvent were added to 3 g. of grains and shaken for the desired length of time on a mechanical shaker. The bottles were centrifuged, the supernatant liquid filtered, and aliquots were analyzed for nitrogen by the micro Kjeldahl method with a mercuric oxide catalyst.

Table I records the pH of the extracts, and the percentage of nitrogen peptized during a 1-hour shaking period. Water, 10% aqueous sodium chloride, 0.075 *N* hydrochloric acid, or 0.01 *N* sodium hydroxide peptize less than 10% of the grain nitrogen, while glacial acetic acid, 90% formic acid, or 0.1 *N* sodium hydroxide disperse up to 35% of the nitrogen. Increasing the strength of the alkali to 0.5 *N* peptizes a maximum of 52% of grain nitrogen.

Water does not extract materially more nitrogen by lengthening the shaking time from 1 to 4 hours (Table II), but 0.5 *N* sodium

TABLE II

EFFECT OF LENGTH OF SHAKING TIME ON NITROGEN PEPTIZATION OF SPENT BREWERS' GRAINS WITH WATER OR 0.5 *N* SODIUM HYDROXIDE

Solvent used	Time of shaking	Meal nitrogen extracted
		%
Water	1	2.9
Water	2	3.2
Water	3	3.2
Water	4	3.3
0.5 *N* Sodium hydroxide	1	52.0
0.5 *N* Sodium hydroxide	2	57.9
0.5 *N* Sodium hydroxide	3	62.2
0.5 *N* Sodium hydroxide	4	68.0
0.5 *N* Sodium hydroxide	6	74.4
0.5 *N* Sodium hydroxide	16	90.6

hydroxide as the solvent raises the nitrogen dispersion from 52% at 1 hour to 74.4% after 6 hours, and to 90.6% after 16 hours' contact time.

Spent brewers' grains, as they reach the market, are of a coarse texture. Ball-mill grinding of the grains for 8 hours at 70 r.p.m. permits a more efficient nitrogen peptization. With 0.5 *N* sodium hydroxide as the solvent 74.4% of the nitrogen is dispersed in 1 hour, and 93.0% after 5 hours shaking (Table III).

The percentage of nitrogen which is removed by high speed centrifugation (about 3,500 r.p.m.), followed by filtration of the supernatant solutions of 0.5 *N* sodium hydroxide extracts of ball-mill-ground grains, after pH adjustment with hydrochloric acid, is shown in Table IV. Two regions of minimum solubility are evident, one at or near pH 3.75 and a second at or near pH 4.55. A maximum of about 75% of the dispersed nitrogen can be recovered at either pH after

TABLE III

EFFECT OF PARTICLE SIZE OF SPENT BREWERS' GRAINS ON NITROGEN PEPTIZATION WITH 0.5 *N* SODIUM HYDROXIDE FOR VARYING EXTRACTION PERIODS

Extraction time	Nitrogen peptized from meal	
	Original sample	Ball-mill ground
Hours	%	%
1	52.0	74.1
3	62.2	83.5
4	68.0	87.3
5	—	93.0
6	74.4	—

an overnight flocculation of the precipitate, followed by centrifugation and filtration of the supernatant liquid. The two solubility minima probably indicate the existence of at least two protein fractions, each with a different isoelectric point. The closeness of the two isoelectric points suggested the use of salts at a pH value half way between the pH minima in order to increase protein recovery. Sodium chloride at pH 4.1, in 30% concentration, did not raise nitrogen recovery; however, either sodium sulfate or magnesium sulfate ($\cdot 7\ H_2O$) in 30% concentrations precipitates an additional 10% of the extracted nitrogen, i.e., a total of 85%.

The precipitate which forms after pH adjustment of the extracts is slimy, and while it packs reasonably well in 50 ml. cups at high centrifugal speeds (about 3,300 r.p.m.) it does not pack in 500 ml. cups at lower speeds (2,000–2,300 r.p.m.). Filtration instead of centrifugation requires frequent changes of filter paper because the slimy film which

settles on the filter paper quickly prevents further passage of liquid. Consequently, when an attempt was made to isolate the precipitated product from a large-scale extraction, yields were much poorer than one would surmise from the theoretical extraction and precipitation data shown in Tables III and IV. An analysis was made of the precipitate obtained from 0.5 *N* sodium hydroxide extracts of ball-mill-ground spent brewers' grains after the addition at pH 4.1 of 30% sodium sulfate. Only 10.2 g. of dried material (calculated on an ash-free and

TABLE IV

EFFECT OF pH ON NITROGEN PRECIPITATED FROM 0.5 *N* SODIUM HYDROXIDE EXTRACTS OF BALL-MILL GROUND SPENT BREWERS' GRAINS

Substance added	pH of solution after 16 hours standing	Extracted nitrogen precipitated
		%
Hydrochloric acid	3.5	63.4
Hydrochloric acid	3.7	74.9
Hydrochloric acid	3.75	75.8
Hydrochloric acid	3.85	70.8
Hydrochloric acid	4.0	68.8
Hydrochloric acid	4.2	67.6
Hydrochloric acid	4.35	69.6
Hydrochloric acid	4.55	74.7
Hydrochloric acid	4.6	71.4
Hydrochloric acid	4.8	50.9
AFTER SALT ADDITION [1]		
Sodium chloride	4.1	75.1
Sodium sulfate	4.1	85.5
Magnesium sulfate ($\cdot 7\ H_2O$)	4.1	85.6

[1] Each salt was added in sufficient quantity to yield a final concentration of 30%.

moisture-free basis) was obtained from 100 g. of spent grains, while theoretically 69 g. should have been the yield $\left(\frac{(4.13)(0.93)(0.855)(100)}{4.75}\right)$. After the addition of salts these unfavorable characteristics of the extracts are further accentuated.

The precipitate was dried under infrared light. A slightly hygroscopic, dark tan powder was obtained. It contained 36.8% ash and 16.4% moisture, the latter determined by further drying of a sample in an Abderhalden vacuum pistol over phosphorus pentoxide at the temperature of boiling toluene. The product contained 69.3% carbohydrate (2), 4.75% nitrogen, no cystine (6), 0.65% methionine (3), and 8.55% glutamic acid (5) [3].

[3] The glutamic acid analyses by the decarboxylase method were performed by Murry Seidman of this laboratory, for which grateful acknowledgment is made.

Discussion

The poor solubility of the nitrogenous constituents of spent brewers' grains could be a characteristic property of the original constituents of the grains, which have been modified by malting and mashing. More likely, however, the lack of solubility is a result of the customary drying methods of either passing hot air through the wet grains, or of exposing them to rotating steam coils. These are ideal conditions for protein denaturation. It is surmised that if drying conditions could be devised which would avoid prolonged exposure of the wet grains to elevated temperatures, the proteins of the spent brewers' grains would probably lend themselves more readily to industrially applicable extraction and purification procedures, similar to those now used on oilseed meals. Whether drying of spent brewers' grains under such conditions is economically feasible is, however, doubtful.

The drastic conditions of ball-mill grinding, relatively high alkalinity of the solvent, and prolonged contact periods of the grains with the solvent, necessary to peptize the nitrogen of commercial spent brewers' grains, also favor the dispersion of the other plant cell constituents. The net effect of the attempted purification is therefore an insignificant raising of the nitrogen content of the isolated "protein" from that of the starting material, namely 4.75% against 4.13% nitrogen, respectively. The complete absence of cystine in the isolated product does not necessarily indicate the absence of cystine in intact spent brewers' grains. It appears more likely that cystine with its known sensitivity to alkali was destroyed during the long exposure to 0.5 *N* sodium hydroxide.

Acknowledgment

This investigation was suggested by Dr. M. J. Blish, Supervisor of Protein Research of International Minerals and Chemical Corporation. The authors wish to express their appreciation to Dr. Blish for his interest throughout this investigation.

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COMMUNICATION TO THE EDITOR [1]

A Method for Grinding Bread Crumb

SIR:

A method for preparing bread crumb for analysis may prove of interest since to our knowledge a similar procedure has not appeared in the literature.

The high moisture content and soft texture of bread crumb make it difficult to grind or otherwise prepare for analysis. Bread crumb can, however, be quickly cut or broken into pieces approximately 2 cm. square, or smaller, and these can be rapidly frozen at −30°C. in a deep freeze unit. This procedure retards biochemical and biological reactions in the bread and the sample can then be stored until convenient to proceed to the next step.

The frozen crumb is ground on a Wiley mill by mixing it with dry ice (solid carbon dioxide). The mill is first chilled by grinding dry ice and then the mixture of dry ice and bread crumb is put through the mill. By this means bread crumb can be finely ground without appreciable change in moisture content. The method is a convenient, rapid, and satisfactory way of obtaining material for the study of bread-staling rates by the water-absorption and viscosity methods. The residual dry ice quickly evaporates, at room temperature, from the ground sample.

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BOOK REVIEWS

Les Succédanés en Panification (Substitutes in Breadmaking). By H. Nuret and R. Calvel with the collaboration of M. Dubois and the staff of the Laboratoire d'Études et de Controle de la Qualité des Blés Français. 115 pp. Published by the Association of Former Students of the French School of Milling, 16 rue Nicolas Fortin, Paris, France. 1948.

This brochure presents interesting and well-organized data concerning the many problems encountered by French bakers during and since the war as a result of the shortage of wheat and the necessity of using long-extraction flour together with numerous substitutes. Except for the periods of the last world wars and since that time, the bread made in France had an enviable reputation and constituted a large part of the diet of the people. Unlike most of the bread in this country, French bread is made from the basic ingredients of flour, water, yeast, and salt, without the addition of milk solids, malt, sugar, shortening, and yeast foods. In the spring of 1948, the shortage of wheat obliged the Government and the bakers to use substitutes for wheat flour in greater quantities than at any previous time. This state of affairs induced the Government to order a study of the many technical problems caused by such additions.

[1] Received November 26, 1948.

The booklet gives a good description of the three principal breadmaking methods used in France. This description is followed by a chapter outlining the regulations and the composition of flours used in France from November, 1940, to November, 1947. The influence of long-extraction flours (up to 98%) on breadmaking characteristics and the general adjustments needed in procedure are briefly discussed. The main part of the brochure describes results obtained using the following substitutes for wheat flour:

1. Cereals: corn, barley, rye, oats, buckwheat
2. Legumes: peas, soybeans, peanuts, beans
3. Potatoes: mashed potatoes, raw crushed potatoes, potato flour
4. Corn starch, potato starch

Details concerning the effect of each of the above additions, in selected amounts from 10 to 50%, on the procedures and the finished bread as compared with the standard are given in a clear, concise manner. The alveograph was used to evaluate some of the mechanical properties of the doughs and, in addition, data are listed on the protein, ash, fat, and diastatic activity values of the various admixtures. The material is presented in the language of the baker with major emphasis on the adjustments necessary to produce the best bread possible from ill-suited but economically necessary substitutes for wheat flour. The technical data are sound and the booklet can be read with profit by anyone interested in baking.

The baking industry of the United States was faced with many trials during the war, but difficulties with 80% extraction flour and shortages of milk solids, fats, and sugars fade into insignificance when compared with the unsurmountable problems of the bakers of France in trying to produce a palatable loaf of bread. Naturally, with the materials they were forced to use, their bread has been poor. It is hoped that the day is near when the good bread of France will return to sustain her people.

BETTY SULLIVAN
Russell Miller Milling Company
Minneapolis, Minnesota

Test Baking. By J. D. Mounfield. 32 pp. The Northern Publishing Company, Ltd., Liverpool 1, England. 1948. Price, 3 shillings.

The author states in the preface that this booklet on test baking is intended mainly as an aid to those engaged in controlling flour quality in mill or bakery. For this purpose it should prove helpful. There are three chapters, namely, "Planning the Test," "Doing the Test," and "Assessing the Result." There is also an appendix containing brief instructions for the statistical treatment of data.

The author has emphasized the importance of having a definite object in mind when planning a baking test and when evaluating the results. He also calls attention to the fact that most of the manipulations in the baking process and the characteristics of the bread produced are matters upon which opinion can, and frequently does, differ greatly. It is lamented that a means has not been found to measure each separate feature of a loaf mechanically and record the result numerically.

The cereal technologist who has followed the development of the baking test as published in the various issues of CEREAL CHEMISTRY would not have much incentive to read *Test Baking*, except perhaps to become better acquainted with a British point of view. The contrast between the problems faced by test bakers in England compared with North America is stressed, and an explanation is given why a standard baking test has not been developed in England.

It is unfortunate that the only references to the official A.A.C.C. baking test are to an article in CEREAL CHEMISTRY (1934) and to *Cereal Laboratory Methods*. Apparently the author is not aware that this book is now in its fifth edition and that the baking test has been modified drastically with time.

The booklet is published with paper covers, the printing is on a good grade of paper, and the print is easy to read. Several illustrations of bread are included.

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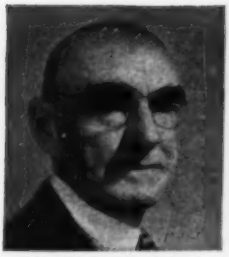

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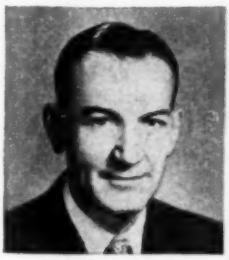

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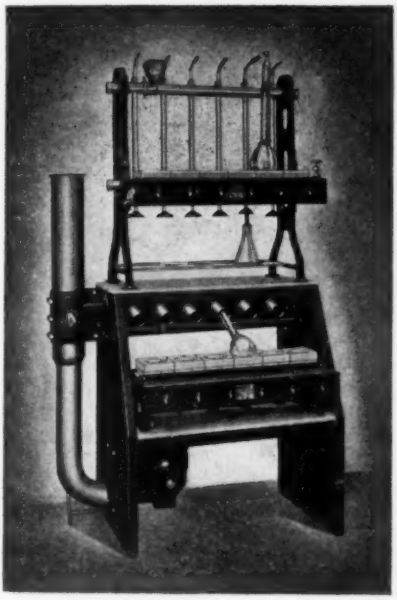

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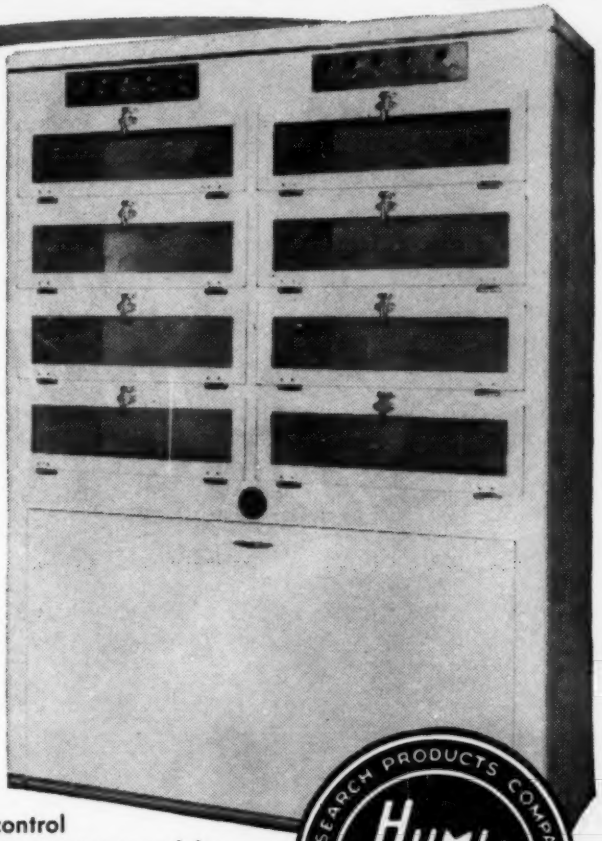

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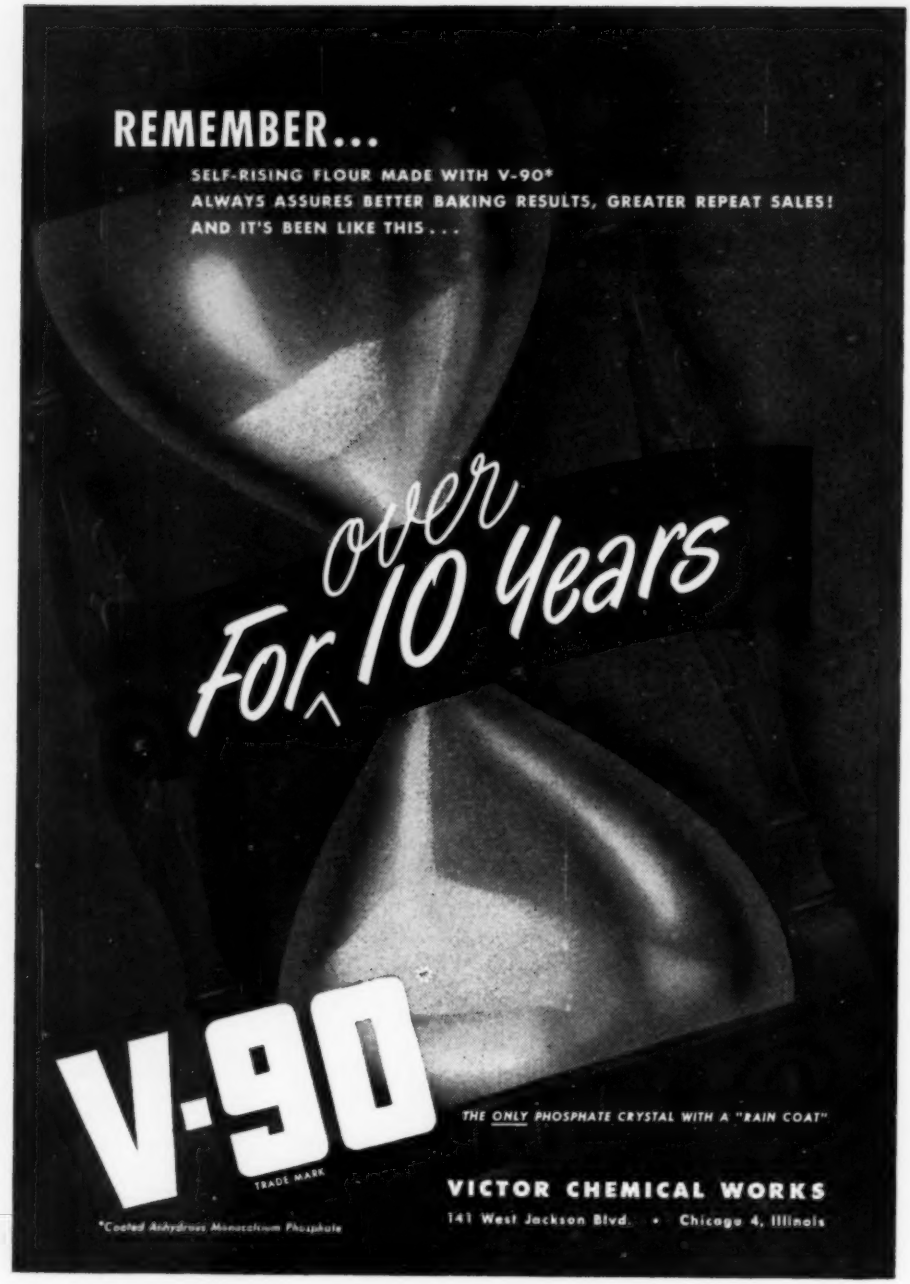
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